

ORAL IMMUNIZATION WITH A RECOMBINANT
SALMONELLA TYPHIMURIUM VACCINE
EXPRESSING A HEMAGGLUTININ OF
PORPHYROMONAS GINGIVALIS

By

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To my parents,
John and Mary Lou Kohler,
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By

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A recombinant, attenuated *Salmonella* vaccine is one approach which induces both mucosal and systemic immunity. The object of these studies was to characterize the immune responses following oral immunization with a recombinant *S. typhimurium* strain, $\chi 4072/pDMD1$, expressing the HagB hemagglutinin of *Porphyromonas gingivalis* and investigate the potential effects of boosting. A "balanced-lethal" construct was employed using an attenuated *Salmonella* strain $\chi 4072 (\Delta asd \Delta cya \Delta crp)$ with the *hagB* gene inserted into a complementary plasmid pYA292 (encoding the *asd* gene).

Induced immune responses following oral immunization with the recombinant *S. typhimurium* strain were determined through a set of studies in BALB/c mice. By ELISA, the kinetics of the primary responses to the *Salmonella* carrier and HagB were shown to be parallel. The serum IgG subclass anti-*Salmonella* and anti-HagB distributions both

represented a Th1-type response, suggesting an additional potential induction of cell-mediated immunity.

The potential for recall responses was also investigated. While a strong recall response followed boosting at week 14, boosting at an earlier time when the primary responses were still strong yielded a reduced response. The inhibition of *Salmonella* colonization of the Peyer's patches due to pre-existing immunity in the gut does not appear to be a possible explanation. Furthermore, pre-existing immunity to the *Salmonella* carrier on subsequent boosting at week 7 or week 14 yielded mixed results. Some reduction in specific anti-HagB responses was shown in serum IgG at week 14 and salivary IgA at week 7; the serum IgA and vaginal IgA response levels were not affected. However, increased responses were shown for all response parameters following boosting at a later timepoint (week 52) which suggests that any inhibitory effect from pre-existing immunity to the carrier is transient.

Other studies investigated long-term responses with boosting at one year. Consistently, strong recall responses, particularly in IgA levels, were shown. These studies demonstrated not only immunological responsiveness in mice at ~57 weeks of age, but also support the notion of long-term mucosal memory.

Specific antibody-secreting cells homing to the systemic and mucosal effector sites were assessed by ELISPOT assay. Specific anti-*Salmonella* spot-forming cells (SFCs) were predominantly IgA. In addition, a significant increase in SFCs was shown following boosting.

CHAPTER 1 INTRODUCTION AND BACKGROUND

The Pathogen *Porphyromonas gingivalis*

Porphyromonas gingivalis (formerly *Bacteroides gingivalis*) is characterized as a gram-negative, non-spore-forming, black-pigmented, anaerobic, rod-shaped bacterium. More notably, *P. gingivalis* has been implicated as an etiologic agent in adult periodontitis (198). Periodontal diseases result in a loss of attachment of the periodontal ligament to the tooth surface and result in bone loss. Periodontal diseases are bacterial infections with recent evidence implicating specific organisms such as *P. gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum*, *Campylobacter rectus* (formerly *Wolinella recta*), *Eikenella corrodens*, and possibly spirochetes (74).

Some recent reports suggest periodontal diseases may present an added clinically significant risk factor for cardiovascular disease (7, 194). In addition, a case-control study of 124 pregnant mothers indicated that periodontal diseases may represent a significant risk factor for preterm low birth weight (160). Together these reports suggest a considerable impact that *P. gingivalis* may have on not only oral but overall health, warranting further investigation of this pathogen.

P. gingivalis possesses numerous adhesin molecules in addition to polysaccharide capsules, cytotoxins, and extracellular hydrolytic enzymes and proteases which aid in its colonization, destruction of the periodontal tissues, and evasion of host immune mechanisms (116, 131). Like other microorganisms that colonize the oral cavity, its adhesins allow for an initial attachment of the organism to specific receptors in an environment continuously bathed by saliva that otherwise would be efficiently removed from the oral cavity. Defined by *in vitro* assays, *P. gingivalis* has been shown to adhere to

saliva-coated and serum-coated hydroxyapatite (196), bind to the protein fibrinogen (117) and epithelial cells (197), coaggregate with several oral gram-positive and gram-negative bacteria (196), and agglutinate erythrocytes from a variety of animals (14, 164, 206).

Hemagglutination, demonstrated *in vitro* as the agglutination of erythrocytes, has been implicated as a potentially important virulence associated factor in other pathogenic bacteria. Several studies have reported the characterization of hemagglutinin proteins isolated from *P. gingivalis* (53, 119, 157, 165, 174) while others have applied molecular genetic studies (118, 172, 173). To date, at least five hemagglutinins (*hagA*, *hagB*, *hagC*, *hagD*, *hagE*) have been cloned and characterized (118, 119, 174). One or more of these hemagglutinins may mediate attachment to host cells.

Protective Immunity to *P. gingivalis*

Many vaccines have been or are being developed against pathogenic organisms known to cause disease. The goal of these various vaccines is to reduce or eliminate the colonization of the known pathogen, interfere with its pathogenic mechanisms and ultimately prevent disease. Likewise, many investigators believe a vaccine targeted against one or more virulence factors of *P. gingivalis*, an implicated pathogenic organism, could reduce or eliminate colonization of deep gingival tissues and prevent further development of periodontal disease. In fact, the critical importance of both innate immunity and T-cell-dependent humoral responses to the prevention of subsequent *P. gingivalis* infection was verified in a recent study utilizing various mouse strains with known alterations in immune system components; normal mice demonstrated a delayed onset and decrease in lesion size of 15-30% compared to the primary infection, while immunodeficient strains appeared unable to develop immune protection to a secondary challenge (102).

At the current level of vaccine development, immunization studies with *P. gingivalis* cannot be performed in humans. However, several useful animal models do exist. In the gnotobiotic rat model, for example, the occurrence of periodontal disease and

recovery of *P. gingivalis* have been reported (62, 89, 106). Other models include the squirrel monkey (*Saimiri sciureus*) (133) and the cynomolgus monkey (*Macaca fascicularis*) (169), the mouse subcutaneous abscess model (11), and the mouse subcutaneous chamber model (73). Various antigen preparations from *P. gingivalis* have been used in immunization studies including whole bacterial cells (27, 28, 108, 133), partially purified fimbriae and purified 43 kD fimbriillin (61, 63, 161), a purified outer membrane preparation (11), and a hemagglutinin (51, 163).

Continuing development of a periodontitis vaccine has focused on a response that would prevent colonization and thus target the infection at an early stage (107). While passive immunization by an oral rinse could effectively prevent exogenous *P. gingivalis* from establishing a niche in the dental plaque, it would not significantly interfere with proliferation of subgingival *P. gingivalis*. A more effective approach would stimulate active synthesis of antibodies in serum and saliva and thus protect the mucosal surfaces and subgingival regions of the oral cavity. Furthermore, a vaccine against *P. gingivalis* may need to confer protection against several of its virulence factors and therefore warrant a combination vaccine.

Mucosal Immunity

While most successful vaccines to date (i.e., rubella, measles, pertussis, meningococcus, and *Haemophilus influenza* type b, among others) have been systemic vaccines that induce serum IgG antibodies and are capable of neutralizing microorganisms or toxins, the response is effective only after the specific organism has breached a mucosal barrier and become systemically invasive (115). In truth, the mucosal tissues serve as primary portals of entry for most infectious diseases, such as HIV-1 (136). Consequently, the focus of current vaccine development is to stimulate long-lasting antibody responses at mucosal surfaces and thereby provide protective immunity against microorganisms that either infect or enter the host through mucosal membranes.

The mucosal tissues that line the gastrointestinal, urogenital, and respiratory tracts constitute a surface area of $\sim 400 \text{ m}^2$; an enormous surface area when compared to the total surface area of skin which is only $\sim 2 \text{ m}^2$ (136). In addition, an estimated 10^{12} organisms per gram of feces make up the normal flora of the intestine, exceeding the total number of cells in the body (58). These bacteria not only help prevent colonization by external pathogens but also prime the estimated 10^{10} lymphocytes/ meter generating cross-reactive antibodies to bacterial antigens (58). The major immune effector molecule present in mucosal secretions is the secretory IgA (sIgA) molecule. It has been estimated that a normal individual makes $>5 \text{ g}$ of IgA daily, representing 65 - 90% of all antibody isotypes produced, thereby making it clearly the predominate Ig isotype in humans (136, 144). In addition, immunohistochemical studies of mucosal tissues and glands have established that the sIgA is locally synthesized by plasma cells found in abundance in mucosal tissues and secretory glands (143). In fact, it has been argued that the intestine is a major lymphoid organ on the basis that 25% of its cellular make-up consists of lymphoid cells (58).

Many studies have focused on further defining the parameters and components of the mucosal immune system (see review (58, 114, 137, 153)). Like the systemic immune system, the mucosal immune system is complex and can be divided into two general sites: the inductive and effector sites (105, 138). Specific inductive sites include areas where antigens are first encountered, processed, and presented with subsequent triggering of specific B and T-cells. Examples of these specialized inductive sites include the Peyer's patches of the gut-associated lymphoreticular tissues (GALT), the palatine and pharyngeal tonsils of the nasal-associated lymphoreticular tissues (NALT), and large intestinal follicles of the urogenital mucosal tissues. The inductive sites are equipped with specialized cells, capable of antigen uptake, which are termed follicle-associated epithelium (FAE) or M cells (12). As reviewed by McGhee and Kiyono (136), microorganisms or antigens are sampled and passaged intact in endocytotic vesicles by these M cells and delivered to underlying lymphoid cells for further antigen processing and presentation. One organism in particular,

Salmonella, exploits this feature and specifically uses these M cells as a portal of entry (166).

Separate from the initial inductive sites, effector sites are areas of mucosal surfaces and glands where these activated immune cells ultimately accumulate and function due to specific interactions with receptor molecules on cells located at these sites (referred to as “homing”) (58, 97). The effector sites include the lamina propria of the gastrointestinal tract, the upper respiratory tract (URT) and the genitourinary tract, as well as the exocrine glands. Unique to these sites is the selective active transport of polymeric IgA into external secretions which has been extensively studied and reviewed (15, 16, 144, 156, 212). Briefly, a specific receptor, pIg, expressed on the surface of epithelial cells interacts exclusively with polymeric, J-chain-containing IgA released from the sub-epithelial plasma cells (16). This complex is then internalized by the epithelial cells, transported in a vesicle to the apical surface of the epithelial cell, where it is then fused with the apical membrane (114, 137). This active transport results in the release of a cleaved portion of the pIg receptor, termed the secretory component (SC), as a SC-IgA complex constituting a secretory IgA (sIgA) molecule in an external secretion (156).

It has also been demonstrated in several experimental models that antigenic stimulation at a local mucosal surface or secretory gland resulted in the production of specific antibodies, not only at this localized site of induction but also in the circulation and in remote external secretions (16, 58, 114, 144, 170). This phenomenon has been referred to as the “common mucosal immune system” (134). The ability to induce secretion of specific IgA at multiple mucosal sites following immunization with an antigen at a single mucosal inductive site has an incredible potential for global protection against a pathogenic organism at the mucosal surfaces.

Secretory IgA has several functional advantages (19, 114, 177). First of all, polymeric IgA has been shown to be more effective than monomeric IgA in neutralizing viruses (209). In addition, sIgA has an added resistance to proteolysis due to its

association with the SC (103). Inhibition of microbial adherence and prevention of absorption of antigens from mucosal surfaces has also been associated with sIgA (103). Another functional feature associated with sIgA is immune exclusion. Through this mechanism, IgA limits further absorption of undigested antigenic material and the potentially harmful formation of circulating immune complexes. These complexes have been associated predominantly with IgG antibodies as seen in IgA-deficient individuals (34). An important factor in the preservation of the barrier function of mucosal surfaces is the inability of sIgA to activate complement, either through the classical or alternative pathways, thereby preventing the induction of local inflammation and enhanced permeability of the mucosal membranes due to tissue damage.

Mucosal Vaccines

Several approaches have been taken toward the development of mucosal vaccines. One approach extensively used involves the use of soluble and particulate antigens in animal models. With the exception of cholera toxin (92), HA of influenza virus (8), and a few others (145) the general observation has been that higher and repeated administrations of oral doses are required for induction of immunity, unlike with systemic immunization. It is believed that the response to oral delivery of antigens is low because most of them are degraded by enzymes secreted in the gut or only partially absorbed due to their high molecular weight. One additional measure that has been used to preserve the immunogenic material from the acid pH of the intestinal environment and proteolysis has been to co-administer large amounts of sodium bicarbonate with the oral antigen.

As mentioned, cholera toxin (CT) which is produced by *Vibrio cholerae* has proven to be one exceptionally effective oral antigen (127). CT has a high affinity for binding to a specific receptor, GM1 ganglioside, found on various nucleated cells including intestinal epithelium. CT is composed of two subunits, A and B. CTB, composed of five identical subunits, is directly involved in the binding of the toxin to cell surfaces, while CTA is the

enzymatic subunit involved in the toxigenic ADP-ribosylation of the adenylate cyclase regulatory protein Gs (138). This results in increased cellular cAMP levels and the influx of cellular secretions (138), yielding the characteristic diarrhea and fluid loss. The CTB subunit has also been found to be a potent adjuvant for use with poor oral immunogens that alone had resulted in oral tolerance (59). Several other investigators have confirmed the adjuvant activity of CT with a variety of other antigens (42, 88, 112, 171, 178, 186). In addition, a recent study determined CTB to directly induce an up-regulation of TGF- β 1 activity and thereby promote the IgA isotype switching and IgA mucosal immunity (104).

Adding to the variety of experimental systems using liposomes to target drugs and antigens by the systemic route, liposomes have recently been considered for oral delivery of antigens (148). In early reports, these small, submicron liposomes containing purified carbohydrate from *Streptococcus mutans*, for example, demonstrated a better induction of an IgA response in orally immunized rats than the carbohydrate itself (147). Similarly, various antigens have been incorporated into biodegradable microspheres (55, 57). These microspheres containing antigen are composed of co-polymers of glycolic and lactic acids which degrade into original catabolizable components by hydrolysis. Eldridge et al. have demonstrated increased antibodies to a *Staphylococcal* enterotoxin following oral immunization with the antigen within a microsphere compared with soluble antigen alone (56). More recently, two novel adjuvants, an oil-in-water microemulsion and a polymeric microparticle, have been developed and reported (158).

A more effective induction of both local and generalized secretory and systemic immune responses has been reported with the use of live attenuated viruses (183) and bacteria expressing a variety of heterologous antigens (136, 137, 141-143). Currently there are two live viral vaccine delivery systems approved for human use: adeno and polioviruses. The trivalent oral (Sabin) poliovirus vaccine (OPV) together with the earlier inactivated (Salk) polio vaccine (IPV) has resulted in the virtual elimination of endogenously transmitted paralytic disease caused by wild poliovirus in the United States

and in many other parts of the world (143). Other attenuated viral vaccines being investigated include respiratory syncytial virus (RSV) (29, 98), rotavirus (86, 159, 218), adenovirus (168, 201, 217), rabies virus (185), human immunodeficiency virus (HIV) (20, 123), influenza virus (78, 207), and vaccinia virus (17, 47, 72, 96, 122, 139, 208, 223, 226). These attenuated vaccines are designed to either induce protective immunity to the wild type strain or serve as recombinant carriers for expression of heterologous genes from target pathogens. Aside from the progress of viral vaccine development, one limitation of this approach is that the introduced recombinant virus may never be cleared from the immunized host and could revert to a wild-type or a completely new strain.

A long-standing approach has been the use of bacteria and their products in mucosal immunizations. In fact, Gay first reported on local immunization with bacteria in 1924 (71). Presently, there are over 17 important mucosal and systemic pathogens that have been used for mucosal immunization (143). *Salmonella* species have been, by far, the most studied recombinant vaccine for the heterologous expression of a variety of pathogenic virulence factors (13, 33, 35, 40, 43, 51, 52, 64, 68, 79, 83, 84, 90, 93, 109, 121, 155, 175, 176, 204, 221, 224). Other important recombinant vaccine carriers include *Vibrio cholerae* (92, 110, 181, 205), *Shigella* species (5, 30, 85, 111, 121), and *Streptococcus* species (46, 140). In all these cases, the live attenuated bacterial strain offers a safer approach than an attenuated virus as the bacterium do not persist, but rather are cleared from the host shortly after immunization.

Recombinant *Salmonella typhimurium* Vaccines

As mentioned, *Salmonella typhimurium* has received much attention and investigation as a recombinant vaccine carrier. Several characteristics of live attenuated *Salmonella* constitutes advantages for its use as a mucosal vaccine (22, 94, 141). First, *Salmonella* naturally targets the specialized M cells of the Peyer's patches in the gut, a

major inductive site of the GALT. In fact, *Salmonella* has even been reported to induce M-cell formation in germ-free mice (189). Likewise, *Salmonella* has been reported to interact with eucaryotic membranes to trigger internalization into non-phagocytic cells through induction of selective aggregation and internalization of host cell surface proteins (70). The multiplication of the live *Salmonella* leads to a strong humoral immune response.

Salmonella are also facultative intracellular parasites, capable of survival in macrophages, and thereby can also induce cell-mediated immune responses (49). In addition, live *Salmonella* expressing a heterologous gene product provides an economical, stable *in situ* synthesis of an antigen. Thus, a recombinant *Salmonella* overcomes two obstacles associated with preparation and oral delivery of a purified antigen: excessive time and cost to purify the antigen, and enzymatic degradation of the antigen while passing through the harsh gastric environment. Furthermore, a live *Salmonella* strain could potentially express multiple gene products simultaneously in one vaccine. In some cases, expression of multiple gene products and subsequent induction of immunity to several putative virulence factors may be necessary to result in effective protection against a pathogenic organism. Many pathogens have been shown to possess multiple virulence factors and in some cases duplication of functions such as adhesins, for example, through several related genes. *S. typhimurium*, for example, has been shown to express multiple fimbrial adhesins. Elimination of one fimbrial adhesin in a mutant strain has been shown to result in no significant reduction in virulence (214). Likewise, *P. gingivalis* has been shown to express multiple hemagglutinins which may function collectively for adherence and may warrant a vaccine approach with expression of multiple gene products.

To optimize the efficacy of a live recombinant vaccine, a variety of mutations have been developed to attenuate the *Salmonella* species. Initially random undefined mutations were employed. However, for development as a human vaccine, a defined set of mutations was desired. The use of temperature sensitive (TS) mutants was attempted. Unfortunately, it was found that TS mutants could grow in mice in areas of lower body

temperature and cause subcutaneous lesions (95). Several other mutations have been developed for attenuation (38, 141, 191). For example, a *strA^d* mutation results in a dependency on streptomycin. On the other hand, a *galE* mutant which specifically affects the UDP4-galactose epimerase results in a sensitivity to galactose *in vivo* (77, 94). Likewise, *pur* mutations (i.e., *purA* and *purE*) have been shown to render the strain attenuated through deficiencies in purine metabolism enzymes (135). Other *Salmonella* strains which have been attenuated through mutations in the prechorismate pathway (25, 91) are referred to as *aro* mutants, and were first described from studies in *S. typhi* (3). These mutant strains do not produce chorismate, an essential intermediate in *de novo* synthesis of aromatic compounds including aromatic amino acids, and are therefore rendered auxotrophic for compounds not available in animal tissues. Other means of attenuation include deletions in *phoP* and *phoP^{*}* which have a global effect on the expression of virulence genes (149, 150). Similarly, a global effect of attenuation has been reported with deletions in the regulator genes *crp* and *cya*, which have global effects on cyclic AMP levels and subsequent inhibition on expression of genes involved with transport and breakdown of catabolites (36, 37). In addition, deletional or insertional mutations have been engineered in the genes *pagC* (involved in capacity to survive in macrophage)(151), *htrA* (encoding a heat shock protease)(26), and *cdt* (associated with colonization of deep tissues)(225). Finally, combinations of these mutations have been engineered to insure stable attenuation with a low probability of a revertant wild-type *Salmonella* in the host. One comparative study of several of these mutant *Salmonella* strains in mice suggested that the level of total Ig specific for the carried antigen is determined by the carrier mutation and the background of the *S. typhimurium* strain (50). More recently, it was reported that the level of protective immunity in mice was dependent on the degree of colonization by the immunizing strain when several combination-mutation *Salmonella* vaccine strains were compared (225). Another approach for attenuation includes curing of the identified “virulence plasmid” on the basis that genetic analysis of

virulence genes encoded on the virulence plasmids of *S. typhimurium* have a direct effect on increasing its growth rate (80, 81).

With regard to developing the actual expression of heterologous genes by these attenuated live *Salmonella*, several important aspects of molecular biology have been considered and investigated. A direct approach for introduction of heterologous gene(s) into *Salmonella* has been the use of a genetically engineered plasmid encoding the gene(s) of interest into the attenuated strain. In so doing, considerations have been made including the stability of the expression system (namely the plasmid), the choice of promoters together with the gene copy number which will determine the level of expression, the toxicity of the gene product to *Salmonella*, and finally the folding and native immunogenicity of the gene product (39, 192). Antibiotic resistance markers on expression plasmids, a common approach for *in vitro* studies in bacterial strains, combined with the use of the equivalent antibiotic in the media provides a selective pressure to insure stable maintenance of the plasmid within the recombinant bacterium. However, for vaccine strains, continued use of antibiotic pressure during immunization is not a favorable approach. Consequently, Curtiss et al. designed a “balanced-lethal construct” deletional mutation in the *asd* gene of a *Salmonella* strain (154). The Δasd mutation is lethal to the gram-negative bacterium in the absence of media containing diaminopimelic acid (DAP) as it is rendered unable to synthesize β -aspartate semi-aldehyde dehydrogenase, a critical step in cell wall synthesis. Without DAP, the cell is unable to create the crosslink bridges of the cell wall structure, resulting in a leaky cell state. Moreover, the mutant is unable to survive *in vivo*. Furthermore, a plasmid was engineered encoding the *asd* gene of *Salmonella* which, when introduced into the Δasd mutant *Salmonella* strain, provides survival of the strain *in vivo* (67). Another approach to overcome the problem of plasmid instability has been reported by Cardénas in which specialized vectors direct the integration of the encoded gene into the chromosome of the mutant *Salmonella* strain (23).

Most promoters used in the recombinant *Salmonella* vaccine experiments, including *lac*, *tac*, and *trc*, had been previously used for molecular cloning and expression studies in *E. coli*. Unlike in *E. coli*, however, the repressors for these promoters are absent in *Salmonella* and therefore are constitutively active (192). While it is speculated that maximal expression of the carrier gene is desirable for optimal immunogenicity, there is also concern that over-expression could be detrimental to the *Salmonella* carrier. A desirable aim, therefore, would be to identify a promoter which is tightly repressed under simple cultivation conditions but would be preferentially turned on in a specific environment within the host. One set of investigators reported the use of an *in vivo* regulated promoter, *nirB*, which is maximally active under anaerobic conditions (24). Subsequently, a different group suggested the superiority of the *htrA* promoter over the *nirB* promoter in an oral vaccination approach (182). Similarly, another group reported a novel approach which would control expression of an antigen whereby the antigen was not expressed *in vitro* but was expressed *in vivo* (60). Briefly, this system utilized two plasmids: one plasmid encoding the *trc* promoter and the heterologous gene, the other a repressor plasmid expressing the *LacI* in trans which would eventually be lost by segregation due to incompatibility with the first plasmid. Yet another approach reported the use of a strong promoter located on a DNA fragment which would invert at random. As a result, the antigen would only be expressed in one particular orientation of the promoter, and, consequently, a bacterial population harboring the plasmid would consist of a sub-population not producing the antigen and therefore would not be affected in growth or persistence in the host (211). The mixed population approach with sub-populations of live recombinant *Salmonella* strains has also been reported recently by Yan and Meyer (222).

Several investigations have focused on the expression of heterologous proteins on the surface of recombinant *Salmonella* cells for optimal immunogenicity. Particularly for induction of immunity to viral or intracellular pathogenic virulence factors, surface expression of the heterologous gene is thought to promote appropriate host responses.

Using polymerase chain reaction (PCR), one group constructed a single-copy expression cassette encoding a chimeric outer membrane protein derived from *OmpA* fused to a truncated derivative of an HIV gp120 antigen (220). They reported surface expression of the *OmpA*/gp120 complex in the recombinant *Salmonella* strain. Similarly, a recent study reported a ten-fold- higher antibody response to a p67 sporozoite antigen (fused to the C-terminal secretion signal of *Escherichia coli* hemolysin) expressed and secreted by *S. dublin* than to a non secreted p67 expressed by the same *Salmonella* strain (75). On the other hand, other investigators have reported earlier that expression of foreign antigen on the bacterial surface is not necessarily essential for induction of appropriate immunity due to the intracellular nature of the *Salmonella* strain (18). It has been suggested that the most important aspect of expression of a foreign antigen for development of an immune response may be the initial amount of antigen that primes the GALT and not persistence of the vector in tissues (22, 33). In other words, very high synthesis of the antigen during the initial phase of invasion could be the most critical element.

In summary, many studies to date have led to further development of live recombinant *Salmonella* as a candidate vaccine vector. In addition, many critical virulence factors required for invasion and colonization of the *Salmonella* in host tissues have been identified. At the same time, other investigators have been developing various recombinant *Salmonella* strains expressing a variety of antigens. Together, these studies have led to further interest and development of live *Salmonella* as a vaccine carrier. Nevertheless, many aspects of *Salmonella* colonization, expression of heterologous genes, and resultant immunogenicity remain a mystery awaiting continued investigations in the future. The focus of these studies was to further investigate the parameters of the host immune response in BALB/c mice resulting from oral immunization with a recombinant *S. typhimurium* expressing a cloned hemagglutinin gene, *hagB*, from *P. gingivalis*.

CHAPTER 2 MATERIALS AND METHODS

Bacterial Strains, Plasmids, Media, and Culture Conditions

E. coli χ 6097 (F⁻ Δ [*lac-pro*] *rpsL* Δ *asdA4* Δ [*zhf-2::Tn10*]*thi* ϕ 80 *dlacZ* Δ M15), a K-12 derivative, *S. typhimurium* χ 4072 (pStSR100⁻ *gyrA1816* Δ *cya-1* Δ *crp-1* Δ *asdA1* Δ [*zhf-4::Tn10*]), an SR-11 derivative, and plasmid pYA292 were provided by Roy Curtiss III (Washington University St. Louis, Mo.)(67). Plasmid pYA292 contains the *asd* gene from *S. typhimurium*, which complements the deletion in χ 6097 and χ 4072, influencing the stable maintenance of the plasmid in the absence of diaminopimelic acid. Plasmid pAX-HagB containing the *hagB* gene of *P. gingivalis* was designed and provided by Ann Progulsk-Fox and Guylaine Lépine (119). Strains were routinely grown at 37°C in Luria-Bertani (LB), SOB-Mg (2% Bacto tryptone, 0.5% Bacto yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl₂, 10mM MgSO₄) or SOC (SOB-Mg + 20mM Glucose) medium as appropriate (188). Media were supplemented, as necessary, with DL- α -diaminopimelic acid (DAP) (50 μ g/ml). Cultures were maintained at -80°C as (20%) glycerol stocks.

Construction of Plasmids

Plasmid pDMD1 containing the *hagB* gene was constructed by inserting the 1.8 kb *hagB* gene from pAX-HagB into pYA292 (Fig. 1) as previously described (52). The orientation of *hagB* in pDMD1 placed it under control of its own promoter.

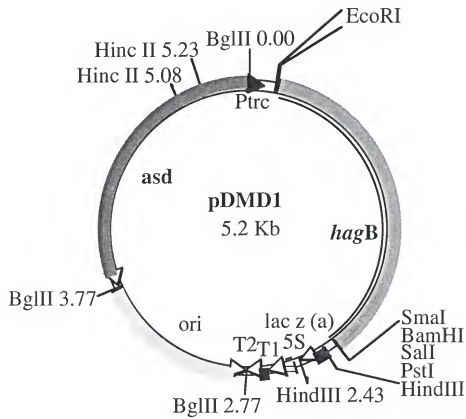


Fig. 1: Plasmid Map of pDMD1.

The recombinant plasmid was transformed into competent cells of the *E. coli* strain χ 6097 by frozen storage buffer (FSB) -based chemical transformation (87). Positive clones were characterized and stored as glycerol stocks as described previously (53).

DNA Characterization

DNA was isolated and characterized by standard techniques (188). From the HagB positive transformant *E. coli* strains χ 6097/pDMD1, one clone was selected and grown on LB medium to isolate plasmid DNA using Wizard Miniprep DNA purification system (Promega, Madison, WI). To verify the presence of a correctly sized *hagB* insert, the plasmid DNA was subjected to restriction endonuclease digestion with *EcoRI* and *HindIII* in a buffered solution for 1.5 hours in a 37°C waterbath. The digested DNA mixture was then electrophoresed through a 1% Agarose gel (SeaKem GTG agarose, FMC BioProducts, Rockland, ME). Plasmid pYA292 linearized with *SmaI* digest, and DNA insert pAX-HagB were added in adjacent wells as positive controls.

Electroporation into *S. typhimurium*.

S. typhimurium strains were transformed by electroporation as described previously (10). Following plasmid pDMD1 purification from the recombinant *E. coli* strain χ 6097/pDMD1, the plasmid was electroporated into the attenuated *S. typhimurium* χ 4072. Competent *S. typhimurium* cells were prepared and stored as a glycerol stocks for electroporation (10). Electroporation was carried out with 2 μ l of DNA (100ng) and 40 μ l of competent cells in a 0.2 cm electrode gap Potter-type cuvette (Bio-Rad Corp., Richmond, Ca.) under the following conditions: 1.25 KV, 400 Ω resistance, and 25 μ f for 9.1 ms. One milliliter of SOC broth with DAP was added and the cells were incubated at 37°C for 1 hour with moderate shaking. The resultant cells were plated with direct, 10^{-1} , 10^{-2} , and 10^{-3} concentrations on SOB-Mg plates with DAP (positive control for viable cells) or without DAP. Positive transformants were identified as growth on selective media

without DAP. Five clones were selected and isolated on separate LB plates for preparation of glycerol stocks and further characterization.

We determined that an extra phenol-chloroform extraction was needed in the Wizard Miniprep plasmid purification from *S. typhimurium* in order to eliminate degradative nucleases. Restriction enzyme analysis, once again was performed with a simultaneous double digestion with *EcoRI* and *HindIII* to verify properly sized insert and vector bands on a 1% agarose gel. HagB expression was detected by SDS-PAGE and Western blot analysis. Quantitation of HagB expression was determined using an enzyme-linked immunosorbent assay (ELISA) as described below.

Lastly, positive clones were tested to check for smooth LPS phenotype using the Tricine SDS-PAGE Silver Staining procedure (120).

Sodium-Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis and Western Blotting

HagB expression of the constructs was determined by sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis of cell lysates. Cell lysates were prepared as previously described (52) and appropriate dilutions of samples were loaded into two duplicate gels of a 4% stacking/ 10% running SDS polyacrylamide gel in a discontinuous buffer system (113). The samples were electrophoresed at 200V for ~40 minutes (Bio-Rad mini PROTEAN II). One gel was stained with Coomassie Brilliant Blue R250 (Bio-Rad) and later dried. The other SDS gel was electrotransferred to nitrocellulose and analyzed for HagB protein using a rabbit IgG anti-HagB absorbed with *S. typhimurium* χ 4072/pYA292, followed by goat anti-rabbit immunoglobulin alkaline phosphatase conjugate (Tago, Burlingame, CA). A lysate of pAX-HagB served as a positive control for each gel.

Colony Immunoblotting To Demonstrate Stable Expression of HagB

Colony immunoblots were performed with a modification of the procedure described by Sambrook (1988). Colonies were transferred to a nitrocellulose membrane and exposed to chloroform fumes for 15-20 minutes to lyse the cells. The membrane was washed briefly with NET Buffer, pH 7.4 (1988), containing 50mM Tris, 150mM NaCl, 5mM EDTA, 0.25% gelatin and 0.05% Triton X-100 and then blocked for an hour with NET Buffer containing 1% Bovine Serum Albumin (w/o Triton X-100). Colonies expressing the *hagB* gene product were detected with the IgG fraction of rabbit anti-HagB absorbed with *S. typhimurium* χ 4072 followed by alkaline phosphatase-labeled goat anti-rabbit immunoglobulin (Tago, Burlingame, CA). The plate was developed with substrate: nitro blue tetrazolium (NBT, 5% in 70% dimethyl formamide (DMF); Sigma Chemical Co.), 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (BCIP, 5% in 100% DMF; FisherBiotech) and $MgCl_2$ (5mM) in Tris-saline (0.1 M, pH 9.6).

Analysis of *in vitro* Growth Rate of Recombinant *S. typhimurium*

The *in vitro* growth rate of the recombinant *S. typhimurium* χ 4072/pDMD1 was studied over a 14 hour incubation under controlled conditions. An overnight static culture of the recombinant strain was prepared from a glycerol stock by inoculation into sterile LB broth and incubation at 37°C. The culture was diluted 1:50 in fresh LB broth and incubated at 37°C in a temperature controlled shaker at 2200 rpm. Aliquot samples were taken from the growing culture at time 0 and subsequently at 30 minute intervals up to the 10th hour and a final time at 14 hours. Optical density (OD) readings were taken for each aliquot with a spectrophotometer of a wavelength of 600nm. Each sample was subsequently plated in serial dilutions on LB plates for enumeration of viable cells for each timepoint. The growth rate study was repeated threefold to verify reproducibility of the recoverable cells with respective OD₆₀₀ readings and timepoints. From the set of data collected, a growth curve

was established with predictable concentrations of cells based on OD₆₀₀ and approximate incubation time.

Mouse Immunization and Sample Collection

Female BALB/c VAF/Plus mice, 6-8 weeks of age, (Charles River, Wilmington, Mass.) were housed in the Infectious Disease Isolation Unit of the University of Florida Animal Resource Center and given food and water *ad libitum*. Generally, groups consisting of six mice were immunized with *S. typhimurium* strain χ 4072/pDMD1. The strain was grown in LB broth overnight at 37°C as a static culture, diluted 1/20 in fresh LB broth, grown 4 hours at 37°C to an optical density at 600nm of 0.8, after which the culture was centrifuged, and resuspended in sterile NaHCO₃ (0.1 M) to a density of 10¹⁰ cells per ml. The mice food supply was removed and the bedding changed 4 hours prior to immunization. Mice were immunized by gastric intubation with 10⁹ cells (0.1ml of 10¹⁰ cells/ml) with a sterile ball-end needle and Becton-Dickinson tuberculin syringe (Aldrich Chemical Company, Inc., Milwaukee, Wis.) in three doses on days 1, 3, and 5 of week 0. Boosting was carried out in the same three dose manner. In colonization studies, however, mice were immunized with a single dose of the recombinant *S. typhimurium* followed by recovery of the bacteria from mouse tissues 5 days later. Serum, saliva, feces, and vaginal washes were among the samples collected for evaluation of specific antibody directed against the hemagglutinin and the *Salmonella* carrier. Mice were anesthetized with sodium pentobarbital (70mg/kg; Butler, Columbus, OH) intraperitoneally and bled from the retro-orbital sinus, collecting 200µl with a heparinized capillary tube. Stimulated saliva was collected using a pipettor after subcutaneous injection of 0.11 ml of sterile pilocarpine nitrate (1 mg/ml ; Sigma, St. Louis, MO). Both saliva and blood samples were centrifuged at 12,000 x g for 15 minutes to remove particulate matter and to separate plasma from the red blood cells, and samples were stored at -80°C. Fecal extracts were processed by suspending fecal pellets (10 volumes w/v) in phosphate buffered saline (PBS)(10mM

sodium phosphate, and 0.85% NaCl, pH 7.4) containing 0.01% sodium azide, centrifuged at 12,000 x g for 15 minutes to remove the particulate matter and the samples were stored at -80°C (45). Vaginal washes were collected by flushing the vagina three times with 80µl of PBS (210). An equal volume of PBS with DTT (0.01M) was added to the washes and incubated on ice for 20 minutes to reduce viscosity before centrifuging the samples at 12,000 x g for 15 minutes. Again, samples were stored at -80°C.

Colonization of Peyer's Patches and Spleen Assay

For the purpose of investigating the colonization potential of the recombinant strain a subset of the group of mice was orally immunized with a single dose of recombinant χ 4072 containing one of several plasmids. Five days later, the subset of mice was euthanized with sodium pentobarbital followed by cervical dislocation. The spleen and a set of five Peyer's patches were surgically removed under sterile conditions. Samples from each mouse were processed separately. Spleen tissues were dispersed in PBS with sterile glass tissue homogenizers and stored on ice. Peyer's patches were dispersed in PBS with a hand-held homogenizer and sterile disposable pestles and stored on ice. Dilutions of each sample were plated on LB agar with nalidixic acid (20 µg/ml). Colony forming units (CFUs) were enumerated and recorded for each sample.

Preparation of Formalin-Killed *S. typhimurium* Cells (χ 4072)

Preparation of whole cell proteins for coating on ELISA plates to determine specific antibody responses to the *Salmonella* carrier was carried out by the formalin-killed procedure (48, 146). Briefly, the *S. typhimurium* strain χ 4072/pYA292 was grown overnight at 37°C as a static culture in SOB-Mg broth (pH 7.5). The culture was diluted (1:50) in fresh media and incubated with shaking overnight at 37°C. The resultant culture was centrifuged at 5000 x g for 20 minutes. The cell pellet was washed (2X) in PBS (0.01M sodium phosphate and 0.85% NaCl, pH 7.4). Then, the cells were resuspended

into 2 volumes of 0.05M sodium phosphate and 0.85% NaCl, pH 7.4, containing 0.056% formalin (1.5 ml/L of 37% stock formaldehyde, Sigma Chemical Co.) and incubated at room temperature with stirring for 90 minutes. After centrifuging the cells and washing (2X) with PBS, the cells were resuspended into 1/2 the starting volume and the cell concentration was determined by the optical density at 600nm. The cell suspension was stored at 4°C with sodium azide added (0.01%). For coating ELISA plates, an aliquot of the cells was diluted in PBS to a concentration of 10^9 cells/ml, centrifuged, and resuspended in the same volume of bicarbonate/carbonate buffer (0.2M, pH 9.6) as described in Immunoassay methods (see below).

Preparation of Sonicated *S. typhimurium* χ 4072/pYA292 Cells

Soluble proteins from *S. typhimurium* χ 4072/pYA292 cells were prepared through a sonication procedure (31). From a glycerol stock (stored at -80°C), an aliquot of LB medium was inoculated and grown as a static culture overnight at 37°C. Diluted in fresh LB medium (1:50), the culture was grown with shaking overnight at 37°C. The culture was then centrifuged at 5000 rpm at 4°C for 20 minutes, and the pellet was then washed (3X) with 0.01M PBS. The washed pellet was resuspended in PBS, concentrated ten-fold from the original overnight volume. The cell suspension was then sonicated on ice with glass beads using a large sonicator (Sonic Dismembrator 300, ARTEK Systems Corp., Farmingdale, NY) at power setting 60 for 8 bursts at 15 second intervals with 5 minutes cooling in between. The resultant sonicated cells were then centrifuged at 9000 rpm for 15 minutes at 4°C. The supernatant was filtered through a low protein binding sterile acrodisc filter (0.2 μ m, Gelman Sciences, Ann Arbor, MI).

The concentration of the protein was determined with a BCA protein assay (Pierce, Rockford, IL). Briefly, the standards and samples were diluted from 2000 μ g/ml to 25 μ g/ml and from direct to 1: 8192 by two fold serial dilutions, respectively. Samples were then placed in designated wells of an ELISA plate in duplicate. Working reagent from the

kit was added to each well, mixed gently, and incubated at 37°C for 30 minutes. The plate was then cooled to room temperature and absorbance measured with MPM titertek (filter with wavelength = 570 nm). MPM program estimated values, the curve fit of the standards and the average value of the protein sample. The protein was further diluted to a concentration of 1.06 mg/ml and aliquots stored at -80°C.

Elispot Assay for Determination of Specific Antibody Secreting Cells

For enumeration of the specific antibody secreting cells from specific effector sites in the mouse, three separate procedures were used for isolation of viable lymphocytes from spleen, lamina propria, and salivary glands. To ensure viability of the cells during the various tissue digestions and separation of cells, the cells were continuously supported in incomplete RPMI 1640 medium (adjusted to pH 7.2 with Bicarbonate/ carbonate buffer and 1mM HEPES) or complete RPMI 1640 media with 10% fetal bovine serum (GIBCO BRL, Grand Island, NY). Tissues were removed from four euthanized mice (as described above) for each trial with sterile surgical technique. Pooled tissue types were processed according to a modification of the following protocols (31, 41, 44, 125) and a communication with Dr. Michael Russell (University of Alabama, Birmingham).

Recovery of viable lymphocytes from the spleens (31) of immunized mice began with suspension of the removed tissues in cold complete RPMI 1640 media in a sterile polystyrene petri dish. Using a sterile mesh screen, the spleens were ground across the screen surface until only a clear membrane remained. The released cells were evenly dispersed and an equal volume of complete RPMI 1640 media was added. The complete suspension was centrifuged for 10 minutes at 4° C at 200 x g. The supernatant was aspirated, and the red pellet was resuspended in cold complete RPMI 1640 medium. Centrifugation and aspiration of the supernatant was repeated. The red pellet was then resuspended in ACK lysing buffer (0.15 M NH_4Cl , 0.1 mM Na_2EDTA , and 1.0 mM KHCO_3 in distilled water adjusted to pH 7.2-7.4 and filter sterilized through 0.2 μm filter

and incubated five minutes at room temperature with occasional shaking to lyse the red blood cells. Afterwards, an equal volume of complete RPMI 1640 medium was added to block the reaction, and the cell suspension was centrifuged. The supernatant was discarded, and the now white pellet was washed twice with complete RPMI 1640 medium and subsequently centrifuged. The remaining viable cells were then resuspended in incomplete media (pre-warmed to 37°C) and purified through a Hystopaque gradient (density 1.083, Sigma) by centrifugation for 15min at room temperature at 800 x g. The resultant pellet was resuspended in complete RPMI 1640 media and stored on ice for subsequent trypan blue staining and enumeration of viable cells.

Recovery of cells from salivary glands from immunized mice, using a modification of a reported procedure (41) and a communication with Dr. Michael Russell (University of Alabama, Birmingham), involved surgical removal of the sublingual and submandibular gland pairs from each mouse. The pooled sets of glands from four mice were collectively weighed and then minced with a sterile scalpel. The tissue fragments were transferred into incomplete RPMI 1640 medium (pre-warmed to 37°C) and a small magnetic stirbar was added. The tissue was then digested with hyaluronidase (14 U/mg, Sigma Chemical Co. St. Louis, Mo), collagenase type IV (2.5 U/mg, Sigma Chemical Co.), deoxyribonuclease I (1016 U/ 4 sets of glands, Sigma Chemical Co.), and dispase (0.05 mg/ ml of medium, GIBCO BRL, Grand Island, NY) in a 37°C waterbath with stirring for 15 minutes. Then the supernatant was aspirated after allowing tissue to settle for 5 minutes without stirring. The digests were repeated an additional two times with fresh enzymes and pre-warmed incomplete RPMI 1640 media for 30 minute incubations. After these last two digests the supernatant was collected, blocked with two volumes of complete RPMI 1640 media and stored on ice. The pooled supernatants were then centrifuged for 5 minutes at 4°C at 300 x g. The supernatant was aspirated, and the pellet was resuspended into incomplete RPMI 1640 media (pre-warmed to 37°C) and purified through a 40% Percoll gradient [4 parts of 100% stock (9 parts Percoll (Sigma Chemical Co.) with 1 part 10X Dulbecco's solution

(Sigma Chemical Co.) in 6 parts incomplete RPMI 1640 with heparin (6000 U/ml)] by centrifugation (2X) at room temperature for 15 minutes at 2300 x g. The purified pellet was resuspended to a final concentrated volume in complete RPMI 1640 media and stored on ice for further determination of viable cell concentration with trypan blue staining.

Recovery of lymphocytes from lamina propria from immunized mice required a modification of another procedure (44, 125). The small intestines from four mice were surgically removed. The intestines were soaked in incomplete RPMI 1640 media. Using a syringe with an intubation needle attached, the intestines were each washed out with incomplete RPMI 1640 medium, removing the fecal contents. On a surgical board, the connective tissues and all visible Peyer's patches were removed from the intestinal surfaces. The cleaned intestines were then cut open longitudinally with a sterile disposable scalpel and then chopped into small 2mm pieces. Collectively the pieces were washed in incomplete RPMI 1640 medium and drained off (6 times). The intestinal pieces were incubated with incomplete RPMI 1640 (pre-warmed to 37°C and containing 5mM EDTA) in a 37°C waterbath with stirring for 15 minutes to release the epithelial cells. After allowing the tissue to settle, the spent media was aspirated off and the incubation was repeated three times with fresh pre-warmed media. The remaining tissue was then blocked with cold complete RPMI 1640 medium for 15 minutes in a 37°C waterbath with stirring. After aspirating off the spent blocking medium, pre-warmed complete RPMI 1640 and collagenase VIII (40 U/ml, Sigma Chemical Co.) was added for stationary digestion for 30 minutes in a 37°C waterbath. The spent medium was collected and stored on ice. Three additional digestions in complete RPMI 1640 and collagenase VIII were carried out, but with stirring. The pooled supernatants were then filtered through glass wool in a glass Pasteur pipette after priming with complete RPMI 1640 medium to prevent attachment of the lymphocytes to the glass surface. The filtered supernatants were centrifuged for 10 minutes at 200 x g, and the pellets were resuspended into one volume of incomplete RPMI 1640. The cells were purified again through a 40% Percoll gradient. The final pellet was

resuspended into complete RPMI 1640 medium and stored on ice for determination of cell concentration following trypan blue staining.

Verification and Enumeration of Viable, Isolated Lymphocytes

Putative isolated lymphocytes were first verified by cyto-centrifugation of an aliquot of the final suspension of cells followed by Wright staining and analysis under a high powered microscope. Briefly, each aliquot (~20-100 μ l) was placed by micropipette in one of 10 cells of a cyto-centrifuge (Shandon Elliott, Co.) consisting of a microscope slide (pre-marked with a wax pencil for identification), a poly-resin re-usable cell with a sample chamber, and a blotter paper between the cell and the microscope slide. Once the samples were loaded and locked into place they were centrifuged for 5 minutes at setting 10 (~600 RPM). Afterwards, the microscope slides were recovered and the concentrated cell spot was stained with Wright stain directly for 1 minute, followed by dilution with an equal volume of dH₂O to the point of creating a silver metallic film and incubated an additional 3 minutes. Then the slides were carefully rinsed with dH₂O until a reddish-pink film on the thinner portion of the cellular mass developed. The cells on the slides were then readily identifiable under a microscope.

Using a hemacytometer, the viable cell concentration (# cells/ ml) from each of the three tissues were determined from aliquot samples combined with equal volume of trypan blue stain (0.4%, Gibco BRL) and viewing under a microscope. Cells were then delivered to ELISPOT wells in direct and 10⁻¹ dilution.

Immunoassay Methods

ELISA Assays

Samples were assayed for antibody to HagB using an enzyme-linked immunosorbent assay (ELISA). Each sample well was coated with 0.1 ml of PBS

containing purified HagB protein (1.25- μ g/ml) isolated by nickel affinity chromatography of the 6x-histidine tagged protein using the QIAexpress system (Qiagen Inc., Chatsworth, CA). The plates were blocked with PBS containing bovine serum albumin (BSA, 0.25%, Gibco BRL). Samples were diluted in the blocking solution and incubated overnight at 4°C. After washing, a horseradish peroxidase-conjugated goat anti-mouse anti-isotype antibody (Fisher Biotech, Pittsburgh, PA.) was diluted in PBS (containing: 0.5% gelatin, 0.05% Tween 20, and 1% Bovine Serum Albumin), added to the wells, and incubated for 3 hours at 4°C. Following washing, 0.1 M phosphate buffer, pH 5.0, containing 0.012% H₂O₂ and 0.04% o-phenylenediamine dihydrochloride was added and the absorbance at 490nm was recorded on one of two microplate readers: Titertek Multiscan MC model 340 (Flow Laboratories, Helsinki, Finland), or MPM Titertek model 550 (BioRad, Hercules, CA). Antibody levels were quantitated by comparison with standard amounts of MOPC 315 IgA, or polyclonal mouse IgG as previously described (52). Briefly, standard wells were coated with the appropriate anti-light chain antisera in order to bind dilutions of the immunoglobulin standards. Standard and sample wells were developed with the same anti-isotype conjugate. Data were analyzed by comparison with log-logit transformed standard curves using Microplate manager III (BioRad, Hercules, CA), and results were expressed as nanograms of immunoglobulin per milliliter of sample.

Specific purified goat anti- mouse IgG subclasses IgG1, IgG2a, IgG2b, and IgG3 and the corresponding mouse IgG subclass standards (Southern Biotechnology Association Inc., Birmingham, AL) were used to assess specific mouse IgG subclass response to HagB and *S. typhimurium* χ 4072.

Specific anti-*S. typhimurium* χ 4072 IgG subclass activity was measured against a formalin killed preparation of the whole cells (48, 146). The cells were resuspended to OD₆₀₀ of 0.8 (yielding 1X10⁹ cells/ml in 0.2M bicarbonate/ carbonate buffer, pH 9.6).

Cells were prepared as described previously and 100µl/well was used for coating ELISA plates.

ELISPOT Assays

Select wells of a 96-well ELISPOT plate (nitrocellulose bottom, Millipore Corporation, Bedford, MA) were coated with either purified HagB protein (1.25 µg/ ml), sonicated *S. typhimurium* extract (1.25 µg/ml), or unlabeled goat anti-mouse IgA antibodies (2.5 µg/ml, Southern Biotechnology Associates, Inc.) in PBS and incubated overnight. Wells were blocked with complete RPMI 1640 for at least 1 hour prior to adding the purified lymphocytes from the three tissues. Each sample set of cells was placed in duplicate in wells coated with HagB, *S. typhimurium*, and goat anti-mouse IgA antibodies (positive control) for enumeration of specific antibody secreting cells to the cloned gene and the *Salmonella* vector. The plate was then incubated at 37°C humidified with CO₂ (5%) for three hours. After washing the plate three times with PBS containing Tween 20 (0.05%, FisherBiotech), biotin-labeled goat anti-mouse IgG, or IgA antibodies (1: 500) in PBS containing BSA (0.25%) were added to designated wells and incubated overnight at 4°C. After washing again three times with PBS/ Tween 20, ExtrAvidin alkaline phosphatase (1:2000, Sigma Chemical Co.) in AP buffer was added to all wells and incubated at room temperature for 30 minutes. After one washing with PBS/ Tween 20 and two washings with Tris-saline buffer (0.1 M, pH 9.6), the plate was developed with substrate: nitro blue tetrazolium (NBT, 5% in 70% dimethyl formamide (DMF); Sigma Chemical Co.), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (BCIP, 5% in 100% DMF; FisherBiotech) and MgCl₂ (5mM) in Tris-saline (0.1 M, pH 9.6). The reaction was stopped with PBS/ Tween 20 containing EDTA (20mM) after ~30 minutes or when violet spots were visible. The plate was then separated to allow for proper drying.

Spots were enumerated in each well under a stereoscopic microscope at 20X magnification. Countable spots for each sample in duplicate were averaged and then

divided by the concentration of viable cells (previously determined by hemacytometer counting) added to the well. Final concentrations were adjusted to be expressed as colony forming units (CFU) / 10^6 cells. Specific IgG and IgA anti- HagB and *S. typhimurium* CFU were determined from the spleen. Specific IgA anti-HagB and *S. typhimurium* CFU were determined from the lamina propria and salivary glands when viable cells were recovered.

Amylase Assay

Antibody levels in salivary secretions, namely specific secretory IgA, were standardized by amylase enzyme activity to account for variable dilutions. Briefly, the enzyme activity for each salivary sample was determined by a modification of an amylase assay reported by Bernfeld (9). Samples were diluted 1:10 and 1:20 in dH₂O. A starch solution [0.4g Potato starch (Sigma Chemical Co.), 30 ml of incubation medium (24.2 g Tris, 160 ml of 1N HCl, 29 g NaCl, 110 mg CaCl₂ in 1 liter volume) and 70 ml dH₂O] was dissolved with heating in a microwave oven until clear (approximately 2X 1 minute intervals). An iodine solution [6 ml of 1N HCl, 3 ml of iodine stock reagent (3 g iodine in 1L of 3% potassium-iodide) and 191 ml of dH₂O] was also prepared. The solutions were pre-warmed in a 37°C waterbath for 15 minutes. Aliquots of the diluted samples (25 µl) were individually added to small tubes containing 2.5 ml of the starch solution, vortexed, and incubated for 5 minutes at room temperature. Then each incubation solution (100 µl) was transferred to a large glass tube containing 5 ml of iodine solution and vortexed. The color change was instantaneous and relatively stable. Each end product was then added to separate ELISA wells in duplicate and optical density at 570 nm was taken with the MPM Titertek reader. The individual color values were compared to the blank and standard controls and the concentration determined by the following calculation:

$$(\text{blank} - \text{sample}) \text{ mg/ml} \times 450(\text{constant}) \times \text{dilution} / (\# \text{ of minutes } (5) \times 1000) = \text{mg/ min/ ml}$$

These values were then used to normalize the salivary specific sIgA levels detected for each sample.

Statistical Analysis

Immune response data from different groups of mice with variable times of boost immunizations were compared with the Wilcoxon rank-sum test for levels of anti-*Salmonella* responses. Colonization data was also compared with the Wilcoxon rank-sum test. Logarithms of the anti-HagB antibody data were compared with repeated measures analysis of variance (ANOVA) for each parameter. Multiple comparisons between means was done with Fisher's Least Significant Difference (LSD) method.

CHAPTER 3 DATA AND RESULTS

Reconstruction and Characterization of the Recombinant *S. typhimurium* strain χ 4072/pDMD1 Expressing the *hagB* Gene of *P. gingivalis*

Transformation into *S. typhimurium*:

The recombinant strain *S. typhimurium* χ 4072/pDMD1 expressing the *hagB* gene under its own native promoter was originally constructed by Dr. David Dusek in the laboratory of Dr. Thomas A. Brown (53). Due to loss of immunogenicity, the recombinant *S. typhimurium* strain was reconstructed at the beginning of these studies. The plasmid pDMD1 was then electroporated into a fresh batch of competent cells of *S. typhimurium* attenuated strain χ 4072 as described previously (Chapter 2: Materials & Methods). Five clones were isolated on separate LB plates for use in making glycerol stocks, minipreps for plasmid verification, cell lysates for verification of HagB protein expression, and LPS silver staining analysis for identification of rough or smooth recombinant *Salmonella* strains.

Restriction Endonuclease Analysis of DNA Plasmid

Purification of the plasmid from the *Salmonella* strain with the use of the Wizard miniprep proved to be more complicated than with the well-characterized *E. coli* strains. After several trials, it was discovered that an additional, final phenol/ chloroform wash was necessary in order to eliminate active endonucleases and recover stable, purified plasmid from the *Salmonella* strain. A double endonuclease digestion of the plasmid pDMD1 miniprep confirmed 2 bands on a 1% agarose gel (Fig. 2, lane *b*). One band of ~3.4 kb aligned with the positive control marker of a linear pYA292 plasmid of ~3.5 kb

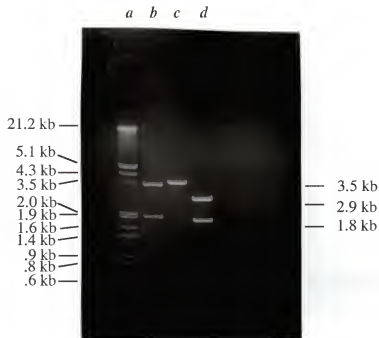


Fig. 2: Restriction digests of plasmid DNA run on agarose gel. Included are the *EcoRI* - *HindIII* digested λ standard (lane *a*), the *EcoRI*- *HindIII* digested plasmid pDMD1 (lane *b*), the *SmaI* digested plasmid pYA292 (lane *c*), and the *HindIII*-*XbaI* digested plasmid pAX-HagB (lane *d*).

(Fig. 2, lane *c*), and the *hagB* gene insert of ~ 1.8 kb aligned with the lower band of the positive control plasmid digest of pAX-HagB (Fig. 2 lane *d*). The slight differences in the bands from the positive controls are explained by the small plasmid fragments from the multiple cloning region that were included in the *hagB* insert or cut from the plasmid band due to the restriction endonucleases used (refer to Fig. 1 of Chapter 2, Materials & Methods).

de novo HagB Protein Analysis

Proteins from cell lysates (prepared as described in Chapter 2: Materials and Methods) were separated by electrophoresis on SDS gels and stained with Coomassie Brilliant Blue. A prominent band was detected for all seven clones corresponding with the positive control HagB protein at 49 kd (Fig. 3). In addition, the identity of the 49 kd band was confirmed to be the HagB protein by Western Blot analysis (Fig. 4). The cell lysates were also analyzed by ELISA to quantitate the HagB expression. The expression level of HagB in the recombinant *S. typhimurium* χ 4072/pDMD1 was 70 μ g/ml of cell culture grown to an $OD_{600} = 0.8$.

LPS Silver Staining Analysis

LPS silver staining analysis was performed as described previously. All five clones demonstrated multiple bands (Fig. 5) indicative of a smooth LPS on the cell surface and a characteristic “smooth” appearance on agar plates. Smooth vaccine strains have been reported to be more immunogenic (100); it is postulated that smooth strains may survive longer in the host due to a reduced ability of immune components (eg. complement) to readily attach to their surfaces.



Fig. 3: Coomassie blue staining of cell lysates. Included are SDS-PAGE low molecular weight standards (lane 1), 7 clones of the recombinant *S. typhimurium* χ 4072/pDMD1 (lanes 2-8), and a positive control, purified HagB protein (lane 9).

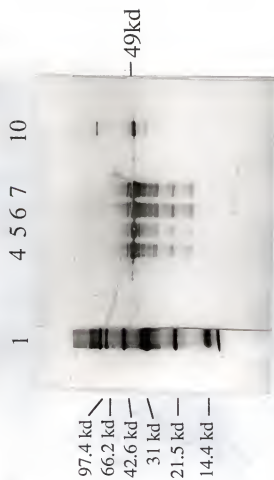


Fig. 4: Western blot analysis for HagB expression. Included are biotinylated SDS-PAGE low molecular weight standards (lane 1), 4 recombinant clones of *S. typhimurium* χ 4072/pDMD1 (lanes 4-7), and positive control_i purified HagB (lane 10). Biotinylated-rabbit IgG anti-HagB absorbed with *S. typhimurium* χ 4072/pYA292 was used for detection of HagB protein.

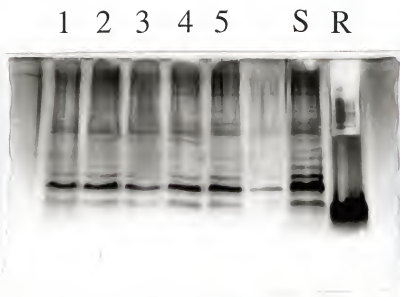


Fig. 5: LPS analysis by Tricine SDS-PAGE and silver staining. Included in the gel are five clones of the reconstructed *S. typhimurium* χ 4072/pDMD1 (lanes 1-5), a smooth, positive control (S) *S. typhimurium* χ 4072, and a rough, negative control (R) *E. coli* strain JM105.

In vitro Growth Rate

The *in vitro* growth rate of the recombinant *S. typhimurium* $\chi 4072/pDMD1$ was analyzed to establish a growth curve (Fig. 6) for the recombinant strain grown at set conditions described earlier (Chapter 2: Materials & Methods). Repeated three times, the growth rates were consistent with a doubling time of ~60 minutes. Earlier studies had established that a dosage of 10^9 cells to be sufficient to induce an immune response in the BALB/c mouse model. Therefore, from the growth curve established from these growth rate studies, a target concentration of 10^8 cells/ ml could be consistently achieved for vaccinations by following the growing cultures to an OD_{600} of ~0.8-0.85. After further concentration of the culture 100:1, the net yield was 10^{10} cells/ ml of which 0.1 ml was orally delivered, or 10^9 cells. The growth curve also revealed that cells used for the oral vaccine were in log phase, which has been recommended for optimum immunogenicity (personal communication with Dr. Roy Curtiss, III).

Colonization Studies of *S. typhimurium* Strains in the BALB/c Mouse Model Following A Single Oral Immunization

In order to determine whether pre-existing intestinal immunity to *Salmonella* could interfere with colonization of a recombinant *Salmonella* strain, several preliminary studies were performed to examine the colonization potential of the recombinant strain of *S. typhimurium* $\chi 4072/pDMD1$ compared to the strain $\chi 4072/pYA292$. Mice were immunized with a single oral dose of one of the two strains using the standard method of preparation and delivery described earlier. The mice were then euthanized with sodium pentobarbital and the spleens and Peyer's patches (average of 5/ mouse) were removed using sterile technique. Initially the sample supernatants were plated in dilutions on MacConkey agar plates (containing bile salts) as the selective media to distinguish *S. typhimurium* from enteric *E. coli* contaminants. From this standard procedure, we had very little recovery of *Salmonella* (data not shown). We hypothesized that transformants

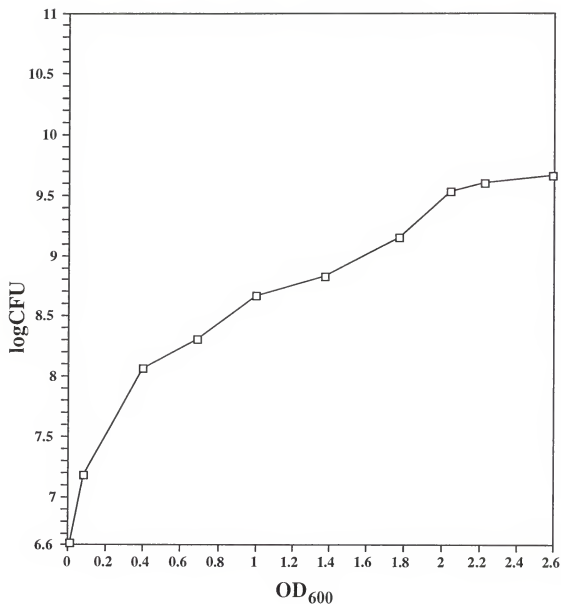


Fig. 6: *In vitro* growth curve for recombinant *S. typhimurium* $\chi 4072/pDMD1$. Representative graph is based on aliquot sampling at time intervals during a growth rate study. Optical density (OD) readings at 600 nm and dilution plating on LB agar plates yielding colony-forming units (CFU) were determined for each aliquot. Growth rate was consistent in three separate studies.

expressing HagB were more sensitive to the selective agents in MacConkey agar. We then investigated the use of less selective media for growth of recoverable cells from the animal tissues. We carried out a comparative study of plating recovered cells on MacConkey agar versus LB agar with nalidixic acid (the $\chi 4072$ *S. typhimurium* strains are resistant to nalidixic acid).

In this study we measured colony-forming units of *in vitro* grown cultures for comparison. Three comparisons were made: viability on LB vs. MacConkey agar, growth of *S. typhimurium* strains $\chi 4072/pYA292$ vs. $\chi 4072/pDMD1$, and re-suspending the cells in sodium bicarbonate vs. PBS (Fig 7). It was determined that MacConkey agar had a limiting effect only on the growth of the recombinant $\chi 4072/pDMD1$ strain. Thus, the overall state of the recombinant strain appears to be weakened by the expression of the heterologous protein and growth on a selective medium such as MacConkey agar further reduces the viability. Therefore, to increase the potential for detecting cells recovered in colonization studies, we selected for *S. typhimurium* strains on LB agar plates containing nalidixic acid. Finally, it was determined that re-suspension in sodium bicarbonate vs. PBS had no significant effect on the growth of either strain. Therefore, oral immunization with the recombinant strain re-suspended in 0.1M sodium bicarbonate directly to buffer the acidic gastric environment would most likely not alter the viability of the vaccine if used within a relatively short time period.

In addition, preliminary studies were conducted to determine the optimal time to attempt recovery of *S. typhimurium* cells from BALB/c mice following a single oral immunization. Recovery of viable cells at 3, 5, and 7 days following oral immunization was tested. From these studies (data not shown) recovery of cells from Peyer's patches at 5 days following the oral immunization was found to be the best time point. For all time points, recovery of viable cells from the spleen were very low or not detectable. It should be noted that the strain $\chi 4072$ lacks the virulence plasmid and therefore this finding of a lack of deep tissue invasion (ie. spleen colonization) is not surprising. So, for comparison

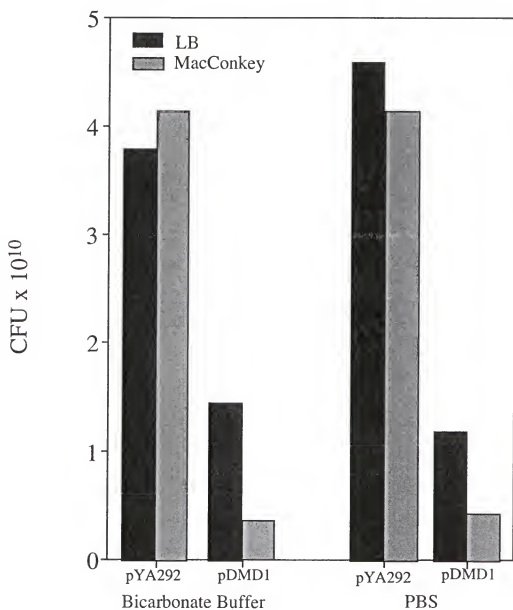


Fig. 7: Recovery of viable *S. typhimurium* χ 4072/pYA292 or χ 4072/pDMD1 cells on LB vs. MacConkey agars. Following *in vitro* growth to an OD₆₀₀ ~ 0.8, both strains were resuspended in sodium bicarbonate or in phosphate buffered saline (PBS). Average yields from duplicate plating are expressed as colony forming units (CFU).

of colonization potential of the *S. typhimurium* strains, only recovery of CFUs from Peyer's patches are reported.

Once the parameters were optimized for the colonization of *S. typhimurium* strains, a comparative study between the recombinant $\chi 4072/pDMD1$ and $\chi 4072/pYA292$ parent strains was conducted. There was a noticeable reduction in the level of CFUs recovered from Peyer's patches for the recombinant strain expressing the HagB gene (Fig 8). The parent strain $\chi 4072/pYA292$ had a mean ($n=4$) of 1×10^5 CFU while the recombinant strain $\chi 4072/pDMD1$ had a mean ($n=4$) of only 1×10^2 CFU. One explanation for this observation could be the fact that expression of a foreign gene might be inhibiting the ability of the recombinant strain to express other genes that are initiated by *in vivo* signaling and have critical, functional roles to survival of the strain *in vivo*.

Assay for Stable *in vivo* Expression of HagB

Parallel to the colonization studies of the recombinant *S. typhimurium* strain $\chi 4072/pDMD1$, the stability of *in vivo* expression of HagB was also investigated. The recoverable viable colonies on LB agar with nalidixic acid were assayed further by colony immunoblotting (see Chapter 2: Materials and Methods). All colonies were picked from primary Peyer's patch isolation plates and patched onto an LB/ nalidixic acid plate for analysis. All colonies were shown to be positive (100 out of 100) for HagB expression along with the positive control, *S. typhimurium* $\chi 4072/pDMD1$ from glycerol stock (Fig 9). In addition, a negative control, *S. typhimurium* $\chi 4072/pYA292$ was also included to ensure that there was no non-specific antibody binding. While this assay was actually performed on recovered colonies grown *in vitro* and does not necessarily represent *in vivo* expression, the results indicate that even after passage through the mouse (*in vivo*) model, retention of the heterologous gene was stable in the recombinant strain.

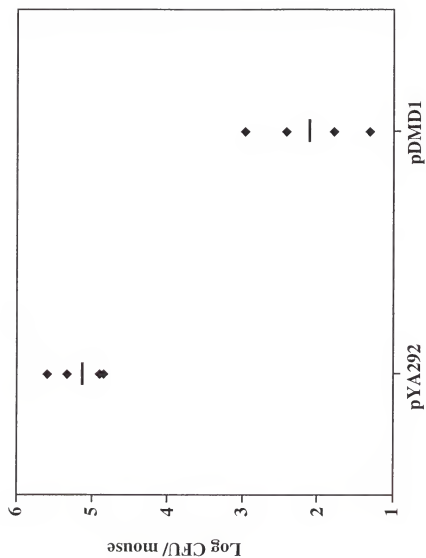


Fig. 8: Colonization of Peyer's patches at 5 days after single oral immunization with *S. typhimurium* strains $\chi 4072/pYA292$ or $\chi 4072/pDMD1$. Presented are individual colony forming units (CFU) from individual mice (diamond) on LB agar with nalidixic acid and the group mean (horizontal bar).

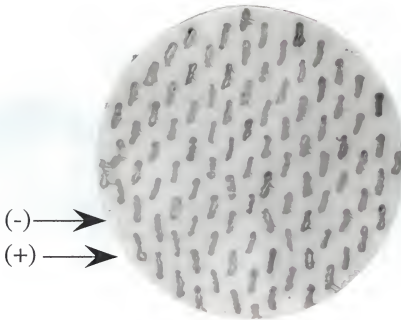


Fig. 9: Colony immunoblot of colonies recovered from Peyers' patches 5 days after a single immunization in BALB/c mice. Colonies were screened for stable production of HagB protein with anti-HagB antibodies. Included were a negative control strain (-) of *S. typhimurium* with pYA292 plasmid only, and a positive control (+) with *S. typhimurium* χ 4072/ pDMD1 from stock .

Oral Immunization Study in BALB/c Mice with Boosting at Week 14

Experimental Design

Previously, it had been reported that a recombinant *S. typhimurium* strain $\chi 4072/pDMD1$ expressing the HagB protein was immunogenic in mice, inducing systemic and mucosal immune responses (52). For most vaccines developed to date, boosting one or two additional times has been found to be necessary for enhancement of immune responses and the development of a potential for long-term memory responses. Due to the diverse compositions of mucosal vaccines developed, conflicting reports would argue whether the potential to induce a long-term memory response exists. In this experiment, we evaluated the ability to induce systemic and mucosal recall immune responses after oral immunization and boosting with the cloned *hagB* gene and examined the IgG sub-class distribution of anti-HagB and anti-*Salmonella* antibodies induced at different stages of the immune response.

The immunization and sampling schedule is provided (Fig 10). Briefly a group of female BALB/c mice (4-6 weeks old) was sampled for serum, saliva, and fecal extracts at week 0, pre-immunization. Mice were orally immunized with three doses of 10^9 cells (prepared as described in Chapter 2: Materials & Methods) on alternate days at week 0. Subsequent samples (including vaginal washes beginning at week 7) were taken at two-week intervals through week 13. In addition to confirming immunogenicity of the re-constructed recombinant strain, the effects of boosting the group of mice with the same recombinant oral vaccine were investigated. The mice were boosted with the same *Salmonella* strain in a similar three-dose manner at week 14 and subsequent samples were taken at two-week intervals. Samples were analyzed for specific anti-HagB or anti-*Salmonella* antibodies by ELISA (described in Chapter 2: Materials & Methods) and group averages with standard error of the mean for each time point were calculated and reported in summary graphs.

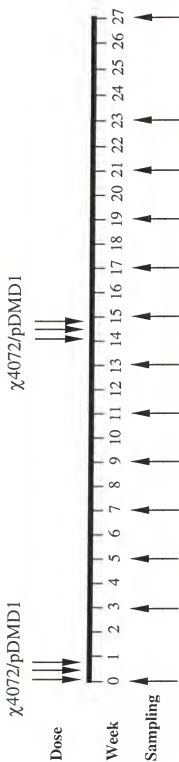


Fig. 10: Immunization & sampling schedule involving oral immunization and boosting of BALB/c mice with *S. typhimurium* $\chi 4072/pDMD1$. Immunizations (downward arrows) were given in three doses on alternate days of week 0(primary) and week 14(boosting). Sampling (upward arrows) of serum, saliva, fecal extracts and vaginal washes (starting at week 7) were collected at alternate weeks.

Detection of Specific Anti- HagB Serum Antibodies After Primary Oral Immunization and Boosting with *S. typhimurium* χ 4072/pDMD1

Mice immunized with *S. typhimurium* χ 4072/pDMD1 developed a strong serum IgG response to HagB (Fig.11). The levels reached a peak at week 5 after primary immunization. The recall response peaked at week 19, five weeks after the boosting at week 14. The mice also developed a primary serum IgA response that peaked at week 7 after primary immunization (Fig. 12). A recall response was also seen at week 19 corresponding to the secondary IgG peak. The serum IgA recall response represented a greater increase over primary than was seen with IgG although the absolute levels were not as high. Both the IgG and IgA responses persisted at high levels throughout the remaining eight weeks of the study. In previous studies, we showed that no response to HagB is detected in mice immunized with *S. typhimurium* containing the cloning vector alone (52).

Detection of Specific Anti-HagB Response In Mucosal Secretions

To assess the mucosal immune response induced to HagB we examined antibody levels in saliva, vaginal washes, and in extracts of fecal pellets. Results were expressed as nanograms of specific IgA antibody per microgram of total IgA in fecal samples and vaginal washes, or per unit of amylase activity in saliva.

A primary peak of salivary IgA anti-HagB was seen at week 9, which was later than that seen for IgG and IgA in serum (Fig. 13). A recall response was seen with a peak at week 17 that was greater than the primary peak at week 9.

Analysis of specific anti-HagB IgA levels in vaginal washes showed detectable levels following primary immunization. Although it was not possible to determine the peak response since data were not available for weeks 0-5, levels appear to decline from week 7 thru 13 indicating the peak has occurred at week 7 or earlier (Fig. 14). Subsequent studies have verified that pre-immunization levels of anti-HagB antibodies in vaginal washes are limited to background. A peak in the recall response was seen at week 17 which was earlier than that seen in serum.

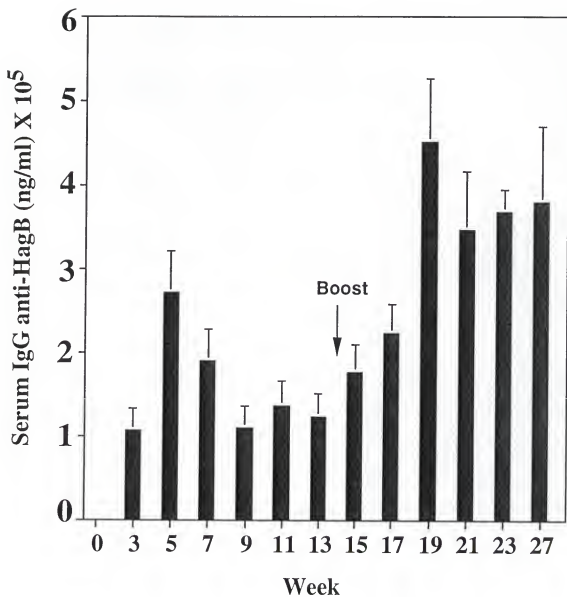


Fig. 11: Serum IgG anti-HagB levels following immunization at week 0 and boosting at week 14 (arrow) with *S. typhimurium* χ 4072/pDMD1. IgG levels expressed as group average ($n = 6$) determined by ELISA of individual samples. Error bars represent the standard error of the mean.

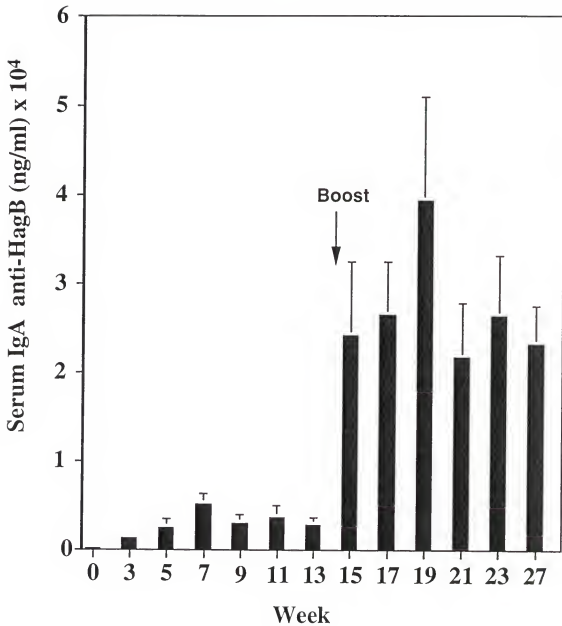


Fig. 12: Serum IgA anti-HagB levels following immunization at week 0 and boosting at week 14 (arrow) with *S. typhimurium* χ 4072/pDMD1. IgA levels expressed as group average (n=6) determined by ELISA of individual samples. Error bars represent the standard error of the mean.

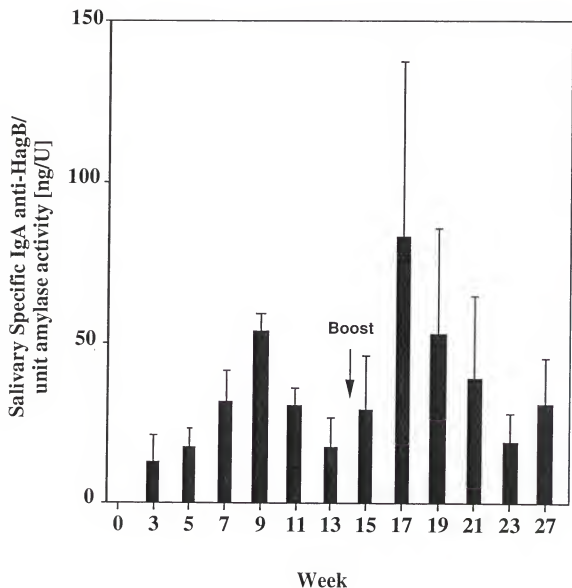


Fig. 13: Salivary IgA anti-HagB levels following immunization at week 0 and boosting at week 14 (arrow) with *S. typhimurium* χ 4072/pDMD1. IgA levels expressed as group average ($n=6$) determined by ELISA of individual samples. Error bars represent the standard error of the mean.

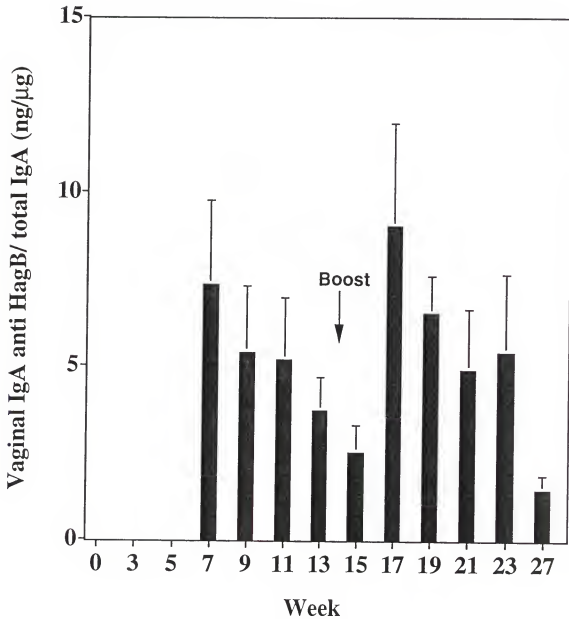


Fig. 14: Vaginal IgA anti-HagB levels following immunization at week 0 and boosting at week 14 (arrow) with *S. typhimurium* χ 4072/pDMD1. IgA levels expressed as group average ($n=6$) determined by ELISA of individual samples. Error bars represent the standard error of the mean.

Fecal pellets were extracted to estimate the level of specific IgA in intestinal secretions. A primary peak was seen at week 7 and a recall peak at week 15, following boosting (Fig. 15). The peak after boosting occurred earlier than that for serum IgG or IgA. There was no pronounced increase in the total IgA in either vaginal washes or fecal samples as was seen in saliva.

Detection of Specific Anti-*Salmonella* χ 4072 Antibodies In Serum IgG and Fecal Extract IgA Levels

High levels of IgG anti-*Salmonella* χ 4072 were found that followed the time course of the specific anti-HagB response (Fig. 16). A primary peak was seen at week 7 followed by a recall response peak at week 19 following boosting. In this case, the recall response was not greater than the primary response. High levels of specific IgA anti-*Salmonella* χ 4072 were also detected in feces (Fig. 17). A primary peak was seen at week 7 was followed by a recall response and peak at week 15 (1 week after boosting) which far surpassed the primary response.

IgG Subclass Distribution Following Primary Oral Immunization and Boosting

It has been reported that the predominance of certain subclasses of IgG reflects either a Th1 or Th2 type helper cell response (202). Th1 responses are associated with a predominance of IgG2a and IgG3 while Th2 responses are associated with IgG1. We examined the serum IgG subclass response at three time points, representing the primary peak, just prior to boost, and the recall peak (weeks 5, 13, and 19 respectively) for both anti-HagB and anti-*S. typhimurium* antibodies.

The anti-HagB response was dominated by IgG2a, which accounted for 70-85% of the response at each time point (Fig. 18). This pattern is consistent with a Th1 type response.

The IgG subclass distribution of antibodies to *S. typhimurium* χ 4072 was also assessed from the serum samples. Serum IgG antibodies specific for *S. typhimurium*

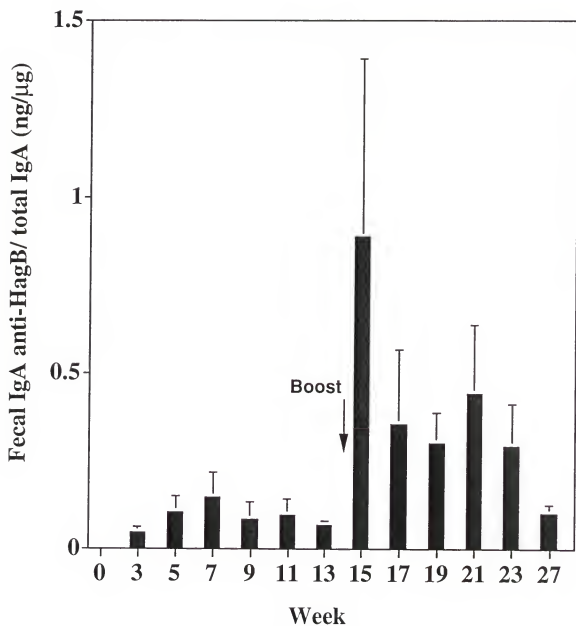


Fig. 15: Fecal IgA anti-HagB levels following immunization at week 0 and boosting at week 14 (arrow) with *S. typhimurium* χ 4072/pDMD1. IgA levels expressed as group average ($n=6$) determined by ELISA of individual samples. Error bars represent the standard error of the mean.

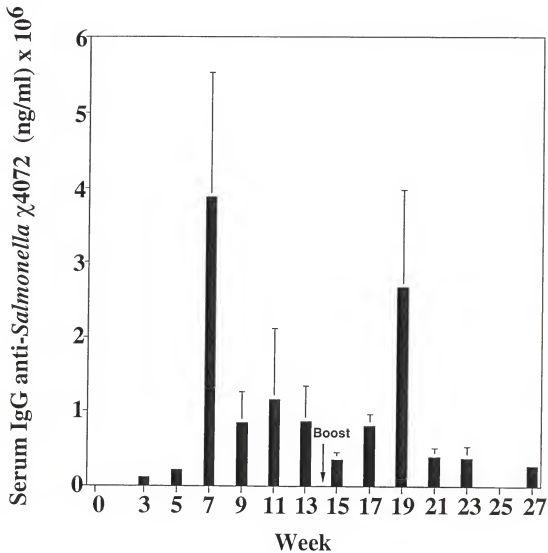


Fig. 16: Serum IgG anti-*Salmonella* levels following immunization at week 0 and boosting at week 14 (arrow) with *S. typhimurium* χ 4072/pDMD1. IgG levels expressed as group average ($n = 6$) determined by ELISA of individual samples. Error bars represent the standard error of the mean.

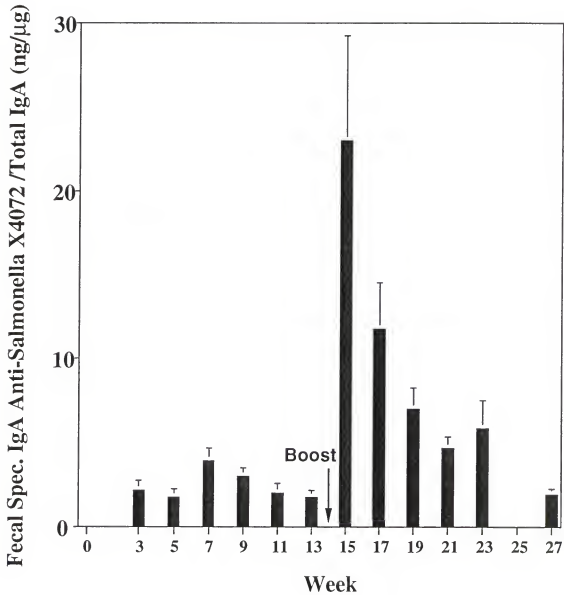


Fig. 17: Fecal IgA anti-*Salmonella* levels following immunization at week 0 and boosting at week 14 (arrow) with *S. typhimurium* χ 4072/pDMD1. IgA levels expressed as group average ($n = 6$) determined by ELISA of individual samples. Error bars represent the standard error of the mean.

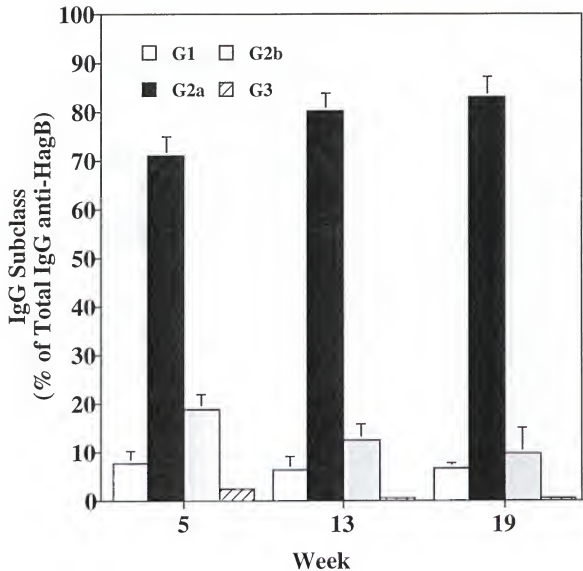


Fig. 18: Serum IgG anti-HagB subclass levels. Time points correspond to: primary (week 5), pre-boost (week 13), and secondary peak (week 19) in mice immunized with *S. typhimurium* χ 4072/pDMD1. IgG subclass levels expressed as percentage (%) of total IgG levels determined by ELISA of individual samples. Error bars represent standard error of the mean.

χ 4072 underwent a shift in subclass predominance (Fig. 19) from IgG3 (70% decreasing to 36%) to IgG2a (17% increasing to 40%) while IgG1 and IgG2b marginally increased (2 to 5% and 10 to 20%, respectively). Again, the absence of IgG1 and the predominance of IgG2a and IgG3 is consistent with a Th1 type response. It is possible that antibodies to O-polysaccharide may have contributed to the IgG3 levels since antibodies to polysaccharides are known to occur in that subclass in mice (195). This supports the findings of others that a *Salmonella* carrier induces a Th1 associated response (18, 32, 187, 193).

Effect of Boosting at Three Different Time Points with Recombinant *S. typhimurium* strain χ 4072/pDMD1 in BALB/c Mice

Experimental Design

Earlier we reported the induction of specific anti-HagB primary and recall responses in serum and mucosal secretions following oral immunization and boosting at week 14 with the recombinant *S. typhimurium* χ 4072/pDMD1. At the same time, we identified responses to the carrier strain, *S. typhimurium* (Figs. 16 & 17). In the area of vaccine development, the practicality of utilizing the same recombinant *Salmonella* strain for boosting or for different vaccines has been a concern because of the response to the carrier strain that could theoretically inhibit colonization in subsequent immunizations. Again, we were able to induce recall responses following immunization at week 14 with the same construct. However, at week 14, the peak of the primary response to the carrier diminished. Therefore, we investigated whether boosting at an earlier time when the response to the carrier was at peak would affect the potential for recall responses.

Specifically, we selected three time points for boosting which would represent an early boosting at week 7 (coinciding with strong primary responses), a later boosting at week 21, and again at week 14 as a control group. The immunization and sampling schedule is shown in Fig. 20. Briefly three groups of female BALB/c mice (6-8 weeks old) were sampled for serum, saliva, and vaginal washes at week 0, pre-immunization.

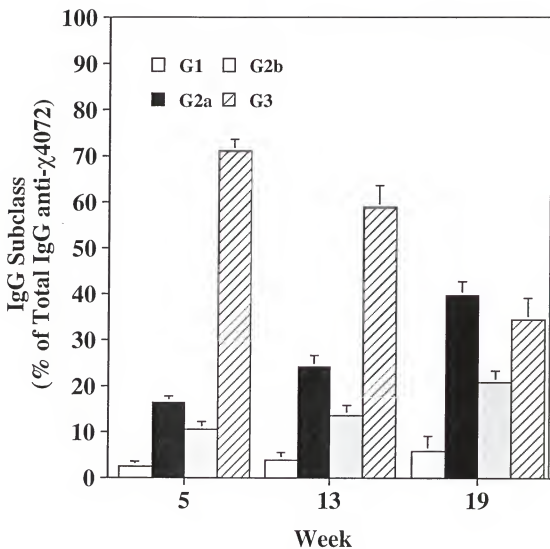


Fig. 19: Serum IgG anti-*S. typhimurium* χ 4072 subclass levels. Time points correspond to : primary (week 5), pre-boost (week 13), and secondary peak (week 19) in mice immunized with *S. typhimurium* χ 4072/pDMD1. IgG subclass levels expressed as percentage (%) of total IgG levels determined by ELISA of individual samples. Error bars represent standard error of the mean.

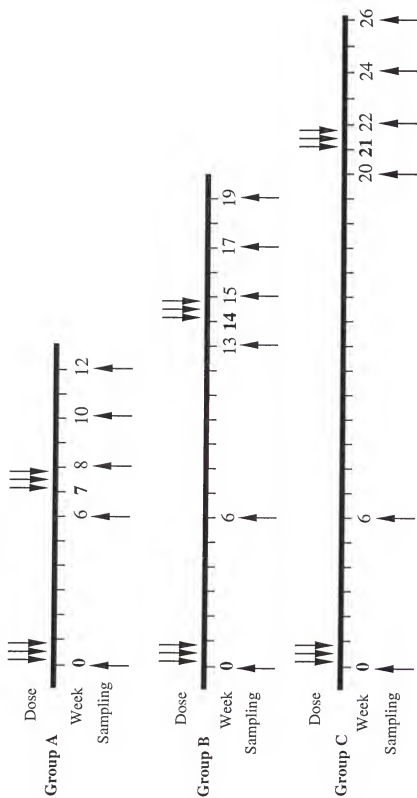


Fig. 20: Immunization & sampling schedule involving oral immunization and boosting at three different times with recombinant *S. typhimurium* χ 4072/pDMD1. Immunizations (downward arrows) were given in three doses on alternate days of week 0 (primary for all 3 groups) and boosting at week 7 (group A), week 14 (group B) or week 21 (group C). Sampling (upward arrows) of serum, saliva and vaginal washes were collected at specific intervals as indicated for each group.

Due to the low level of specific anti-HagB levels detected from the fecal extracts in the previous experiment, fecal extracts were not collected in this and subsequent experiments for detection of specific anti-HagB IgA. Mice were orally immunized with three doses of 10^9 cells (prepared as described in Chapter 2: Materials & Methods) at week 0.

Subsequent samples were taken at selected time points indicated. All groups were boosted with the same *Salmonella* strain in a similar three-dose manner at either week 7, 14, or 21 and subsequent samples were taken in two-week intervals. Samples were analyzed for specific anti-HagB antibodies by ELISA (described in Chapter 2: Materials & Methods) and group means for each time point were calculated and reported in summary graphs.

Detection of Specific Anti- HagB Serum Antibodies After Primary Oral Immunization and Boosting at Different Time points with *S. typhimurium* γ4072/pDMD1

Using the repeated measures analysis of variance, comparisons were made between week 6 and each of the 3 measurements after the boost for each parameter and group. Based on ELISA analysis of the serum samples, we found variation in the amplitude of the recall response means for each group that was a resultant effect of the time of boosting (Fig. 21). Specific anti-HagB IgG for Group I went from a mean of 2.5×10^5 ng/ml at week 6 to a maximum level from the three post-boost sample times of 4.9×10^5 ng/ml (a 2-fold increase from baseline at week 6) at week 10 (3 weeks post-boost). In comparison, the specific IgG response in Group II went from 1.5×10^5 ng/ml at week 6 to 4.1×10^6 ng/ml (a 11- fold increase from baseline at week 6) at week 17 (3 weeks post -boost). Similarly, the specific IgG response in Group III was also increased several fold with a shift from 1.1×10^4 ng/ml at week 6 to 2.3×10^6 ng/ml (a 68-fold increase from baseline at week 6) at week 22 (1 week post-boost).

Specific IgA anti-HagB responses in serum samples were also compared (Fig. 22). Once again, group I averages were lower in magnitude following boost compared to the other groups. More specifically, the average for Group I specific serum IgA was 304

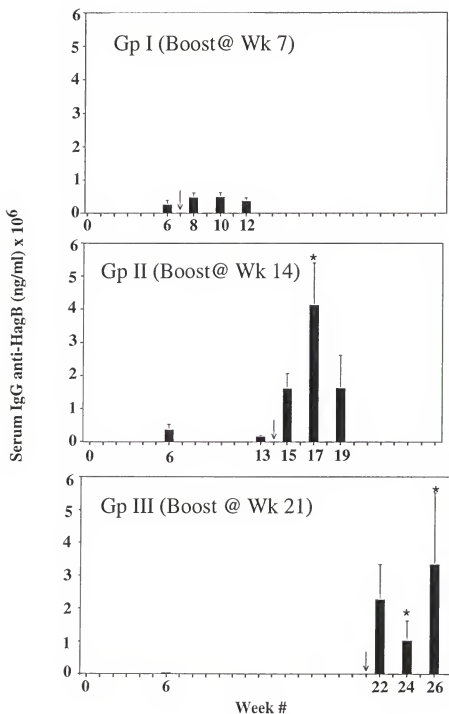


Fig. 21: Serum IgG anti-HagB levels following immunization at week 0 and boosting at week 7 (group I), 14 (group II), or 21 (group III) with *S. typhimurium* χ 4072/pDMD1. IgG levels expressed as group average (n=6) determined by ELISA of individual samples. Error bars represent the standard error of the mean. Statistically significant increases ($p < 0.05$) in IgG levels from the primary response at week 6 are indicated for each group (*).

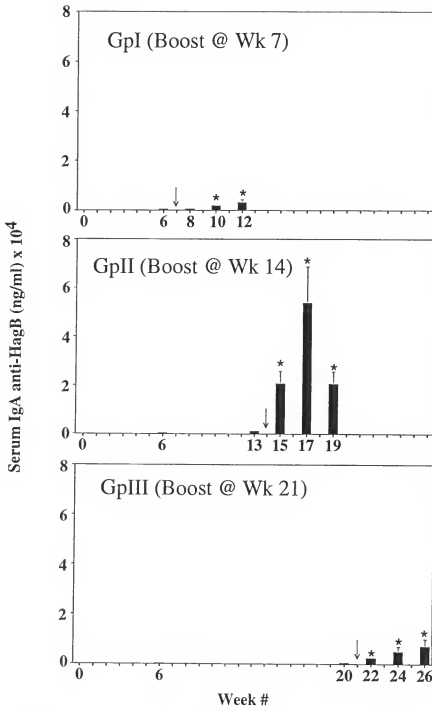


Fig. 22: Serum IgA anti-HagB levels following immunization at week 0 and boosting (arrow) at week 7 (group I), 14 (group II), or 21 (group III) with *S. typhimurium* χ 4072/pDMD1. IgA levels expressed as group average (n=6) determined by ELISA of individual samples. Error bars represent the standard error of the mean. Statistically significant increases ($p < 0.05$) in IgA levels from the primary response at week 6 are indicated for each group (*).

ng/ml at week 6 and only increased to a maximum of 3002 ng/ml (a 10- fold increase from baseline) at week 12 (5 weeks post -boost). Similarly, Group III IgA response averages were also only moderate from 226 ng/ml at week 6 to 7116 ng/ml (a 31- fold increase from baseline at week 6) at week 26 (5 weeks post-boost). In contrast, the specific IgA response averages for Group II were greatly increased and demonstrated a strong recall response with a baseline level of 354 ng/ml at week 6 increasing up to 5.3×10^4 ng/ml (a 152- fold increase from baseline) at week 17 (3 weeks post-boost).

Detection of Specific Anti-HagB Responses In Mucosal Secretions

Analysis of the salivary secretions also showed a difference in specific IgA anti-HagB responses between the groups (Fig. 23). While all three groups demonstrated a recall response following their respective boosts, Group II once again had a higher overall magnitude of response. The salivary specific IgA response for Group I went from 14 ng/ml at week 6 to a maximum average level of 211 (a 15-fold increase from baseline) at week 12 (5 weeks post-boost). The average specific IgA response for Group II was 32 ng/ml at week 6 and increased to 887 ng/ml (a 28-fold increase from baseline) at week 15 (1 week post-boost). The average specific IgA response for Group III went from 3 ng/ml at week 6 to 356 ng/ml (a 118-fold increase from baseline) at week 26 (5 weeks post-boost). Interestingly, Group II had the highest magnitude of specific IgA response but Group III had the greatest fold increase from its respective baseline, which incidentally was the lowest week 6 baseline response of all three groups. Overall, no statistically significant differences were determined between the groups.

Analysis of the vaginal wash samples for the three groups showed average specific IgA levels that were relatively consistent, with recall responses for all three groups (Fig. 24). However, none were statistically elevated over week 6. Specifically, the average vaginal specific IgA response for Group I was 3 ng/ml at week 6 and increased to 13 ng/ml (a 4-fold increase from baseline) at week 12 (5 weeks post-boost). The average vaginal

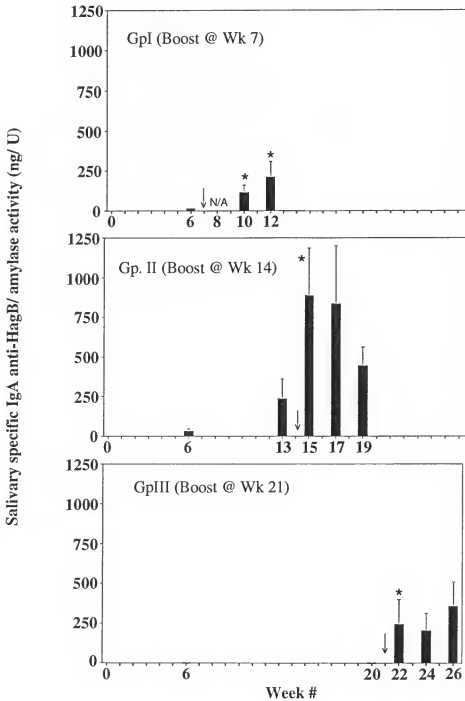


Fig. 23: Salivary IgA anti-HagB levels following immunization at week 0 and boosting (arrow) at week 7 (group I), 14 (group II), or 21 (group III) with *S. typhimurium* χ 4072/pDMD1. IgA levels expressed as group average (n=6) determined by ELISA of individual samples. Error bars represent the standard error of the mean. Statistically significant increases ($p < 0.05$) in IgA levels from the primary response at week 6 are indicated for each group (*).

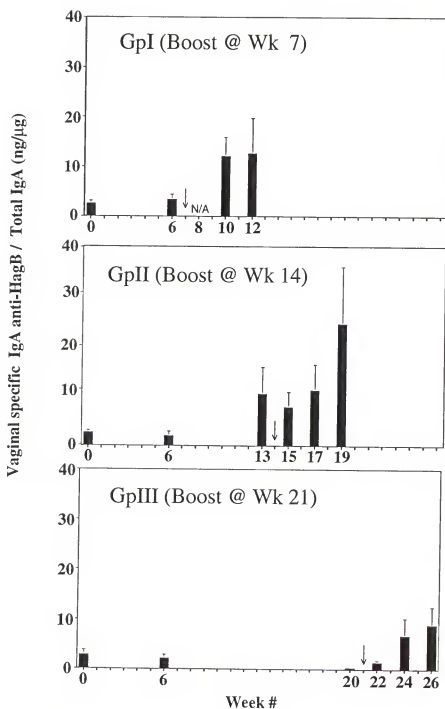


Fig. 24: Vaginal IgA anti-HagB levels following immunization at week 0 and boosting (arrow) at week 7 (group I), 14 (group II), or 21 (group III) with *S. typhimurium* χ 4072/pDMD1. IgA levels expressed as group average (n=6) determined by ELISA of individual samples. Error bars represent the standard error of the mean.

specific IgA response for Group II went from 2 ng/ml at week 6 up to 24 ng/ml (a 13-fold increase from baseline) at week 19 (5 weeks post-boost). Lastly, the average vaginal specific IgA response for Group III was 2 ng/ml at week 6 and increased to 9 ng/ml (a 4-fold increase from baseline) at week 26 (5 weeks post-boost).

Effect of Pre-existing Immunity to the *Salmonella* strain on Subsequent Oral Immunization in BALB/c Mice

Experimental Design

The previous experiment indicated that mice are refractory to boosting at week 7 during the peak of the response to the *Salmonella* carrier. This may be due either to immune interference or lack of sufficient memory at the earlier time. The goal of this study was to directly address the effect of pre-existing immunity on both the immune response to and colonization of $\chi 4072/pDMD1$.

Two groups of mice (n=10) were orally immunized at week 0 with the carrier strain containing the vector, *S. typhimurium* $\chi 4072/pYA292$, to induce pre-existing immunity to the *Salmonella* carrier. Two additional groups were given saline as parallel control groups. The immunization and sampling schedule is shown in Fig. 25. Briefly the groups of female BALB/c mice (6-8 weeks old) were sampled for serum, saliva, and vaginal washes at week 0, pre-immunization. Subsequent samples were taken at selected time points indicated (s) for each group and assayed for specific immunity to *S. typhimurium*. Then, one group previously immunized with the carrier and one control (saline) group was orally immunized with the three-dose regimen of *S. typhimurium* $\chi 4072$ at week 7 or week 14. Subsequent samples were analyzed for specific anti-HagB antibodies by ELISA (described in Chapter 2: Materials & Methods) and group means for each time point were calculated and reported in summary graphs.

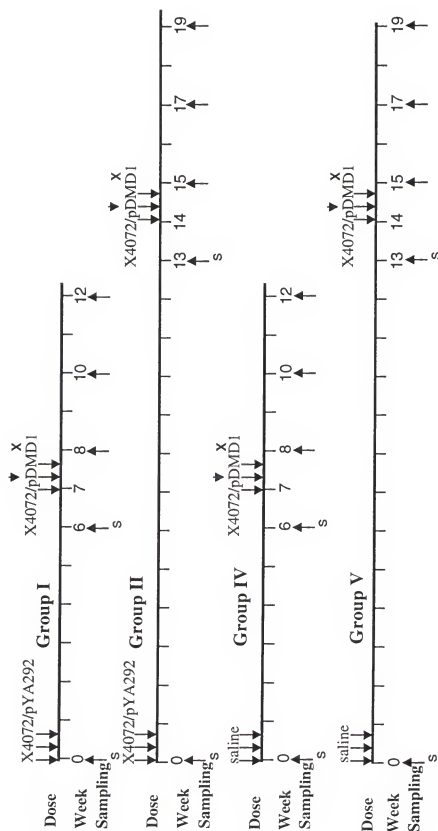


Fig. 25: Immunization & sampling schedule investigating effect of pre-existing immunity to *Salmonella* carrier strain. Immunizations (downward arrows) were given in three doses on alternate days. Sampling (upward arrows) of serum, saliva, and vaginal washes were collected at specific intervals as indicated for each group; where indicated with an S-upward arrow, the samples were assayed for anti-*Salmonella* antibody levels. In addition, a subset of mice (X) were killed at five days following a single immunization (single downward arrow) for recovery colonization studies.

Detection of Specific Anti-*Salmonella* responses Following Oral Immunization with Strain χ 4072/pYA292 at week 0.

Non-immune samples from all four groups of BALB/c mice were collected at week 0 prior to immunization with attenuated *S. typhimurium* χ 4072/pYA292 (Group I and II) or saline (Group IV and V). As anticipated, no background immunity to *Salmonella* components was detected by ELISA for serum IgG or IgA, as well as in fecal IgA, while background levels of specific IgA were detected in all vaginal wash samples. Following primary immunization with χ 4072/pYA292 or saline during week 0, groups I and IV were again sampled and assayed for specific anti-*Salmonella* IgG or IgA levels at week 6, just prior to their immunization with the recombinant *S. typhimurium* χ 4072/pDMD1. As expected, the group orally immunized with *S. typhimurium* χ 4072/pYA292 demonstrated a strong primary anti-*Salmonella* response in serum IgG, serum IgA, fecal IgA, and vaginal IgA levels (data not shown). In contrast, Group IV (saline controls) remained at zero or background anti-*Salmonella* levels in all samples (data not shown). Similarly, Group II and Group V were sampled and assayed for anti-*Salmonella* Ig levels at week 13, just prior to their respective immunizations with the recombinant *S. typhimurium* χ 4072/pDMD1. Once again, Group V (saline controls) had no specific anti-*Salmonella* Ig levels, while Group II had apparent anti-*Salmonella* IgG and IgA levels, although overall levels were lower for Group II at week 13 than for Group I at week 6 (data not shown). This difference in the anti-*Salmonella* Ig levels correlates with our previous studies in which an anti-*Salmonella* response within a group was very strong at week 6 but had plateaued at a lower level by week 13. Statistically, Group I and IV were significantly different ($p < 0.004$) for all response levels. In addition, Group I and Group II (both initially immunized with the *Salmonella* carrier strain) were significantly different ($p < .0001$) for the serum IgA levels.

Detection of Specific Anti- HagB Serum & Mucosal Antibodies After Oral Immunization with Recombinant *S. typhimurium* χ 4072/pDMD1 at Week 7 or Week 14 in Groups With or Without Pre-existing Immunity to the *Salmonella* Carrier Strain.

Following the oral immunization of Group I and IV at week 7 with 3 doses of *S. typhimurium* χ 4072/pDMD1, the two groups were sampled at 1, 3, and 5 weeks post-immunization and assayed by ELISA for specific anti-HagB Ig levels in serum, vaginal washes, and saliva. Values were determined for all three time points post-immunization, however only the peak responses at 5 weeks post-immunization were compared using the Wilcoxon rank sum test.

The anti-HagB IgG response in serum was significantly reduced ($p < 0.05$) in mice pre-immunized with the *S. typhimurium* carrier and then immunized at week 14 (Fig. 26). While there was some reduction in mice immunized at week 7, the difference was not statistically significant. Pre-immunization with the *Salmonella* carrier had no effect on serum IgA anti-HagB levels at either week 7 or 14 (Fig. 27).

With regard to mucosal responses, pre-immunization with the carrier strain significantly reduced the salivary IgA anti-HagB response in animals immunized at week 7 (Fig. 28). Levels were reduced in animals immunized at week 14 but the difference was not statistically significant. No statistically significant differences were seen in vaginal wash IgA anti-HagB levels at either week 7 or 14 (Fig. 29).

Assessment of the Colonization of Peyer's Patches Following Oral Immunization With the Recombinant *S. typhimurium* χ 4072/pDMD1 in Mice With or Without Pre-existing Immunity to the *Salmonella* Carrier Strain.

A subset of each group of mice was assessed for the level of colonization of the spleen and Peyer's patches. Following a single oral dose of *S. typhimurium* χ 4072/pDMD1 at week 7 or week 14, mice were sacrificed and the spleen and an average of 5 Peyer's patches were removed for homogenization. Following plating on selective agar plates, the CFUs were enumerated for individual mice within each group. No CFUs

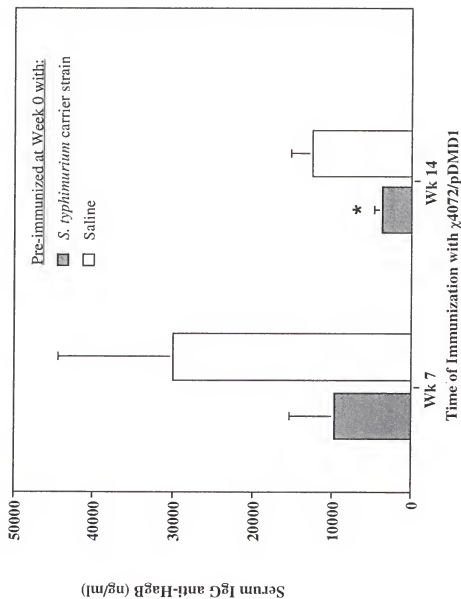


Fig. 26: Serum IgG anti-HagB responses from groups of BALB/c mice to determine effect of pre-existing immunity. Presented are responses at 5 weeks following oral immunization with *S. typhimurium* χ 4072/pDMD1 at week 7 or 14. IgG levels expressed as group averages (n=6) determined by ELISA of individual samples. Error bars represent the standard error of the mean. Statistically significant decreases ($p < 0.05$) in IgG levels in groups previously immunized at week 0 with *S. typhimurium* χ 4072/pYA292 versus the corresponding control group (saline) are indicated (*).

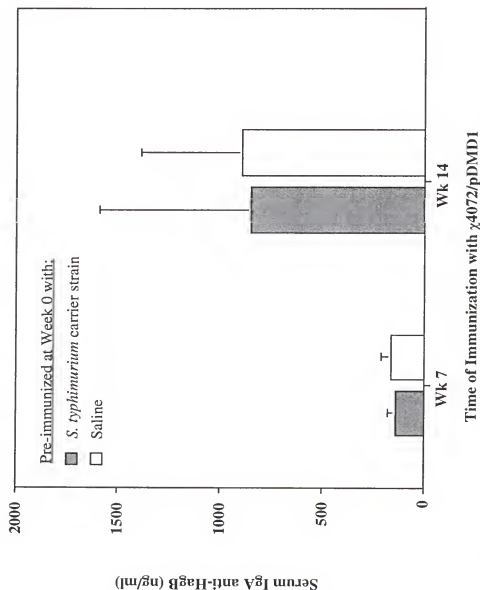


Fig. 27: Serum IgA anti-HagB responses from groups of BALB/c mice to determine effect of pre-existing immunity. Presented are responses at 5 weeks following oral immunization with *S. typhimurium* $\chi 4072/pDMD1$ at week 7 or 14. IgA levels expressed as group averages ($n=6$) determined by ELISA of individual samples. Error bars represent the standard error of the mean. Statistically significant decreases ($p < 0.05$) in IgA levels in groups previously immunized at week 0 with *S. typhimurium* $\chi 4072/pYA292$ versus the corresponding control group (saline) are indicated (*).

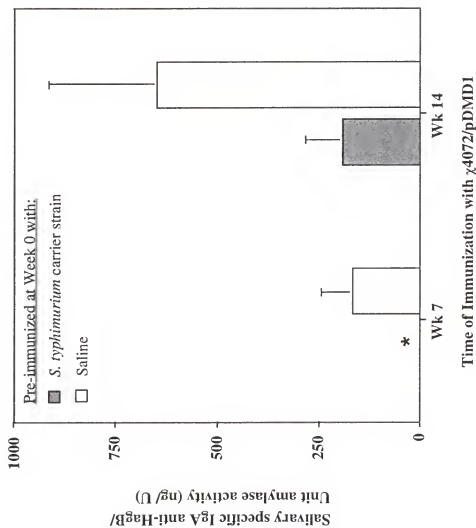


Fig. 28: Salivary IgA anti-HagB responses from groups of BALB/c mice to determine effect of pre-existing immunity. Presented are responses at 5 weeks following oral immunization with *S. typhimurium* $\chi 4072/pDMD1$ at week 7 or 14. IgA levels expressed as group averages (n=6) determined by ELISA of individual samples. Error bars represent the standard error of the mean. Statistically significant decreases ($p < 0.05$) in IgA levels in groups previously immunized at week 0 with *S. typhimurium* $\chi 4072/pA292$ versus the corresponding control group (saline) are indicated (*).

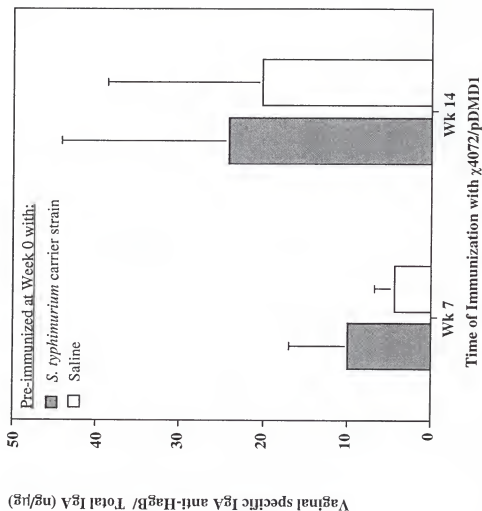


Fig. 29: Vaginal IgA anti-HagB responses from groups of BALB/c mice to determine effect of pre-existing immunity. Presented are responses at 5 weeks following oral immunization with *S. typhimurium* χ 4072/pDMD1 at week 7 or 14. IgA levels expressed as group averages (n=6) determined by ELISA of individual samples. Error bars represent the standard error of the mean. Statistically significant decreases ($p < 0.05$) in IgA levels in groups previously immunized at week 0 with *S. typhimurium* χ 4072/pYA292 versus the corresponding control group (saline) are indicated (*).

were recovered from the spleens of any group. From the Peyer's patches, within each group there was some variability between the individual mice (Fig. 30). However, there was no statistically significant difference between the average CFU/ group; all four groups had an average of $\sim 3 \times 10^4 - 1 \times 10^5$ CFU/mouse. These results demonstrate no inhibition of colonization due to pre-existing immunity to the carrier strain.

Studies Investigating Long Term Memory and Potential for Recall Response Following Boosting at Week 52 (One Year)

Experimental Design

In the previous studies we have shown that oral immunization with *S. typhimurium* expressing a foreign antigen can lead to a recall response shortly after a course of primary immunization (i.e. week 14). The longevity of memory, particularly mucosal memory, induced by oral *Salmonella* carriers has not been adequately demonstrated. In this study we wished to examine whether recombinant oral *Salmonella* $\chi 4072/pDMD1$ could induce a "lifelong" capacity to mount a recall response following challenge. The immunization and sampling schedule is presented (Fig. 31).

Three groups of mice were examined. Group I was immunized at week 0 and boosted at week 52. Week 52 was chosen to represent long-term memory since it equals approximately one-half the lifespan of a BALB/c mouse. Group II was immunized at week 14 and boosted at week 52. Group III was immunized at week 0 and boosted at week 14 as well as week 52. (Note that Group II was immunized in the previous study investigating pre-existing immunity and kept for week 52 boosting in this study to conserve mice. Likewise, Group III was immunized as part of the earlier experiment determining the effect of time of boost on recall responses.)

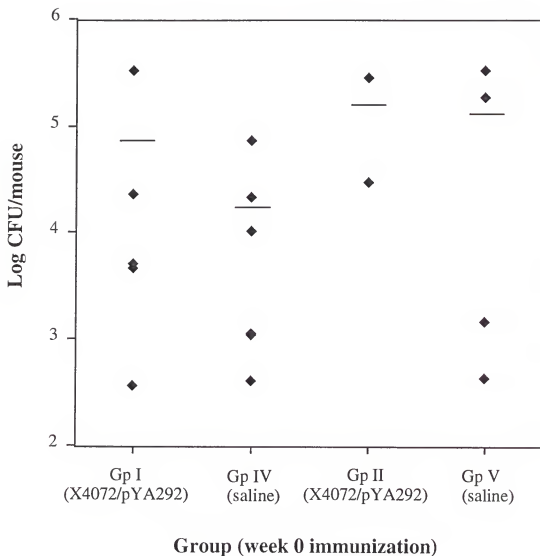


Fig. 30: Colonization of Peyer's patches to determine the effect of pre-existing immunity to the *Salmonella* carrier. Represented are colonies recovered at 5 days following single oral immunization with *S. typhimurium* χ 4072/pDMD1 at week 7 (groups I and IV) or 14 (groups II and V) in groups of mice with prior immunization at week 0 with *S. typhimurium* χ 4072/pYA292 (groups I and II) or saline (groups IV and V). Presented are individual colony forming units (CFU) on LB agar with nalidixic acid from individual mice (diamond) and the group mean (horizontal bar).

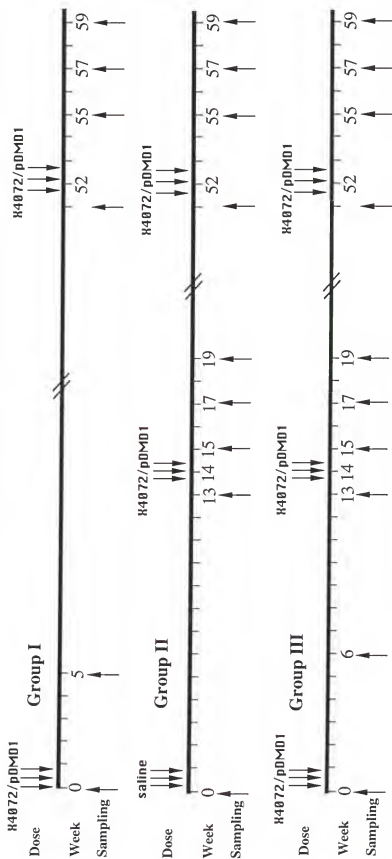


Fig. 31: Immunization and sampling schedule investigating long-term memory and potential for recall responses following boosting at week 52. BALB/c mice were orally immunized with recombinant *S. typhimurium* χ 4072/pDMD1 and subsequently boosted with the same recombinant *S. typhimurium* strain. Immunizations (downward arrows) were given in three doses on alternate days. Sampling (upward arrows) of serum, saliva, and vaginal washes were collected at specific intervals as indicated for each group.

Determination of Specific Anti-HagB Serum Responses Following Boosting at Week 52 in Three Separate Groups of BALB/c Mice

Serum samples collected at the specified time points for each of the three groups were analyzed for specific anti-HagB IgG and IgA responses by ELISA (Figs. 32 & 33). The levels of responses are presented as group means of specific Ig levels. All three groups showed a recall response in serum IgG and IgA as well as mucosal IgA. Group I, immunized at week 0, had very little residual response at week 51 (one week pre-boost), but had a subsequent strong recall response following boosting at week 52 with a serum IgG anti-HagB level of ~180,000 ng/ml (Fig. 32). Although this level of response was only marginally higher than the primary response at week 5 of ~135,000 ng/ml, it was a significant recall response for one year after primary exposure. In comparison, Group II which had been immunized at week 14, had a residual IgG response at week 51 of ~35,000 ng/ml. Following boosting at week 52, the serum IgG anti-HagB response increased to a steady level of ~75,000 ng/ml. Despite an overall lower level of response within Group II, a strong recall response following boosting at week 52 was detected. Group III, previously immunized at week 0 and week 14, again had a low residual IgG response at week 51. Following boosting at week 52, a recall IgG anti-HagB response was observed immediately but was not to the amplitude of the response following the first boosting at week 14. Still, the IgG levels after week 52 were significant at ~750,000, higher than for the other two groups.

More significant were the serum IgA recall responses for all three groups following oral boosting at week 52 (Fig. 33). A strong IgA recall response, ~6,400 ng/ml, for Group I was detected after only 3 weeks following the week 52 boost; this recall response far surpassed the primary response of ~600 ng/ml. Similarly, Group II had an immediate IgA recall response, at 3 weeks post-boost, of ~9,000 ng/ml which was a ten-fold increase from the primary peak response of ~900 ng/ml. Group III had an IgA recall response level at ~43,000 ng/ml following the boost at week 52 which was comparable to the earlier recall

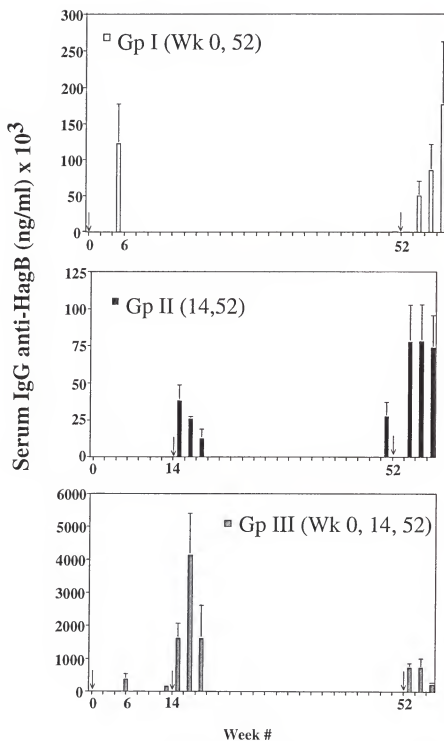


Fig. 32: Serum IgG anti-HagB levels for three groups following various primary immunizations and boosting at week 52. All immunization timepoints are indicated (arrow). IgG levels expressed as group average ($n=6$) as determined by ELISA of individual samples. Error bars represent the standard error of the mean.

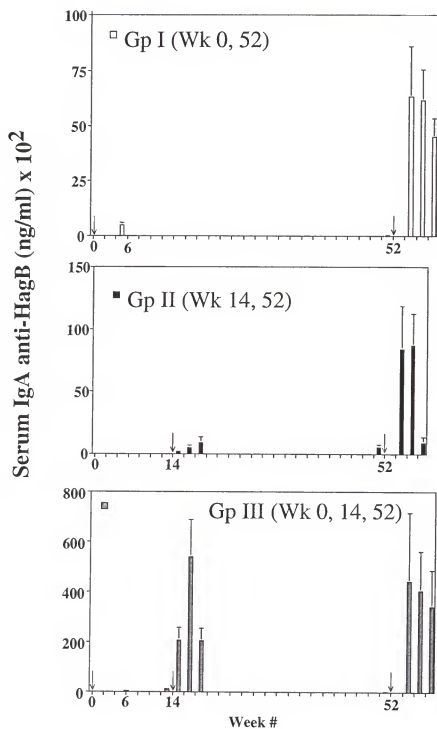


Fig. 33: Serum IgA anti-HagB levels for three groups following various primary immunizations and boosting at week 52. All immunization timepoints are indicated (arrow). IgA levels expressed as group average ($n=6$) as determined by ELISA of individual samples. Error bars represent the standard error of the mean.

response following the first boosting at week 14. Despite no significant increase in the recall level of IgA following the second boosting, the absolute level was approximately nine times greater than the magnitude of the IgA responses in the other two groups.

Determination of Specific Anti-HagB Mucosal Responses Following Boosting at Week 52

Mucosal responses in the three groups following boosting at week 52 were determined by ELISA and presented as means of specific anti-HagB sIgA levels (Fig. 34 & 35). Overall, the mucosal responses demonstrated a similar pattern of characteristic recall responses with earlier (within 3 weeks of boosting) and elevated absolute levels of sIgA. Salivary IgA anti-HagB levels (Fig. 34) were greatly elevated in Group I and III following boosting at week 52 to ~120 ng/U and ~2800 ng/U, respectively. The recall response levels represented a 10 to 20-fold increase over the primary responses. Similarly, Group II demonstrated a marginal increase to ~1000 ng/U following the week 52 boost.

Vaginal IgA anti-HagB levels (Fig. 35) were also reflective of a recall response. Group II and Group III had particular increases in responses following the boost at week 52 with respective levels of ~70 ng/μg IgA and ~100 ng/μg IgA. Group I had a post-boost level that was comparable in absolute concentration to the primary response but appeared within 3 weeks instead of 5 weeks following oral immunization. Surprisingly, the residual primary IgA response at week 51 was still at considerable levels in all three groups.

ELISPOT Analysis of Specific Antibody- Secreting Cells from Spleen, Salivary Glands, and Lamina Propria of BALB/c Mice Following Oral Immunization or Boosting

Experimental Design

Collectively the previous set of experiments has determined the immune responses following oral immunization with respect to concentration of specific immunoglobulin in circulation or secretions. In this set of experiments we set out to define the specific immune response following oral immunization or boosting with the recombinant *S.*

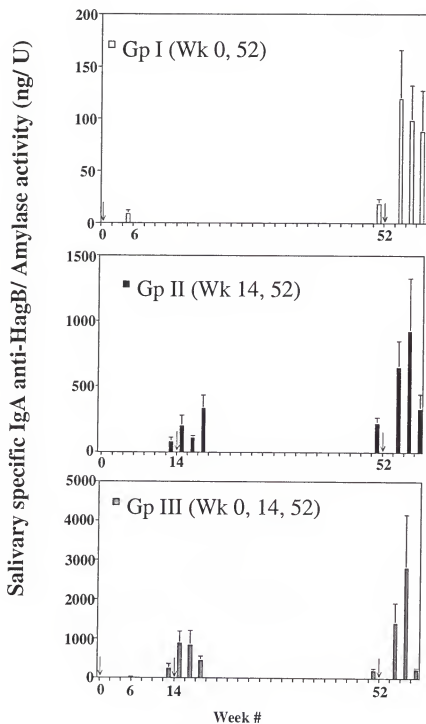


Fig. 34: Salivary IgA anti-HagB levels for three groups following various primary immunizations and boosting at week 52. All immunization timepoints are indicated (arrow). IgA levels expressed as group average ($n=6$) as determined by ELISA of individual samples. Error bars represent the standard error of the mean.

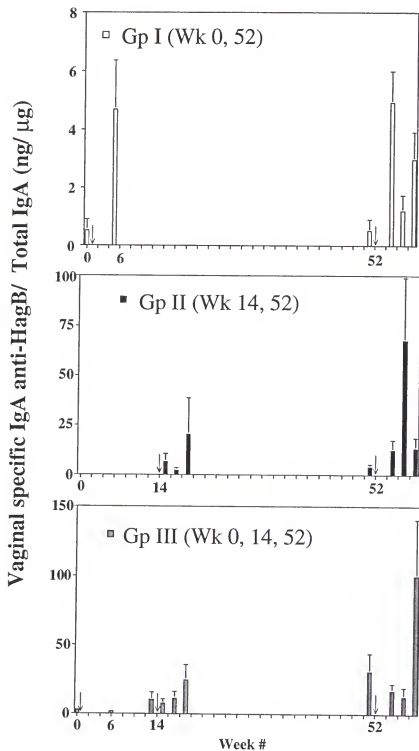


Fig. 35: Vaginal IgA anti-HagB levels for three groups following various primary immunizations and boosting at week 52. All immunization timepoints are indicated (arrow). IgA levels expressed as group average ($n=6$) as determined by ELISA of individual samples. Error bars represent the standard error of the mean.

typhimurium χ 4072/pDMD1 at the cellular level. Specific antibody-secreting cells from spleen, salivary glands, and lamina propria of BALB/c mice were assayed by ELISPOT. In preliminary studies we examined spot-forming cells (SFC) at weeks 1, 2, 3, 4, and 5 following oral immunization (data not shown). From these studies it was apparent that the level of anti-HagB specific spots was low, even at the peak of the response, preventing us from doing a kinetic study. Thus we focused efforts on comparing groups at peak times only. It was determined that 5 weeks post-primary immunization yielded sufficient spot-forming cells from all three tissues. In addition, it was determined that a minimum group of four was required to obtain enough viable cells from the pooled samples for plating.

Collectively, four groups (n=4) of BALB/c mice at five weeks following primary oral immunization and two groups (n=4) at 3 weeks post-boosting (week 14) were assayed. Viable lymphocytes isolated from the spleen were assayed for specific IgG and IgA antibody-secreting cells directed towards *S. typhimurium* χ 4072 and the HagB protein. Similarly, isolated cells from the lamina propria and salivary glands were assayed for specific IgA antibody-secreting cells directed towards *S. typhimurium* χ 4072 and the HagB protein. IgG SFC were not assayed from lamina propria or salivary glands since they are not found in high numbers in these mucosal tissues. The specific antibody-secreting cells were enumerated and reported as SFC/ 10^6 mononuclear cells (MNC). The collective results of these studies are summarized in Table 1.

Enumeration of Specific Spot Forming Cells (SFC)

The average concentration of specific anti-*S. typhimurium* IgG and IgA was 6.5 and 81 SFCs/ 10^6 MNC, respectively. Surprisingly, immune cells stimulated against the *Salmonella* carrier strain were predominantly IgA versus IgG with a ratio greater than 12:1. In contrast, the average concentration of specific anti-HagB IgG and IgA was nearly equal. Most interesting is the contrast in the concentration of specific antibody-secreting cells from the group boosted at week 14. In this group, the number of stimulated cells from the

Table 1. Cells secreting antibodies to *S. typhimurium* and HagB in various organs of BALB/c mice orally immunized with recombinant *S. typhimurium* χ 4072/pDMD1

Cell source	SFC/10 ⁶ MNC secreting:			
	Anti- <i>S. typhimurium</i> IgG	Anti- <i>S. typhimurium</i> IgA	Anti-HagB IgG	Anti-HagB IgA
Spleen (primary)	6.5 $\pm$ 1.8	81.2 $\pm$ 45	8.5 $\pm$ 2.9	6 $\pm$ 3.5
Spleen (boost)	5.3 $\pm$ 0.9	41.8 $\pm$ 3.7	21.4 $\pm$ 6.8	46 $\pm$ 2.1
Salivary gland (primary)	ND	4.5 $\pm$ 2.6	ND	0.5 $\pm$ 0.5
Salivary gland (boost)	ND	42.9 $\pm$ 5.6	ND	67.4 $\pm$ 8.9
Lamina propria (primary)	ND	93.5 $\pm$ 34.5	ND	21.5 $\pm$ 11.5
Lamina propria (boost)	ND	164.5 $\pm$ 68.2	ND	81.5 $\pm$ 9.6

ND= not determined

spleen directed toward the components of *S. typhimurium* was similar to the previous levels in the primary immunized animals. This lack of increase reflects what we observed in serum in terms of anti-*S. typhimurium* IgG levels after boosting (Fig. 16). In contrast, boosting the group of mice resulted in a 4-fold increase in specific IgG and 7-fold increase in specific IgA SFC's directed to the HagB protein.

Spot-forming cells from the salivary glands following primary immunization were difficult to determine due to low abundance and low cell yield from salivary glands. However, following boosting at week 14, the concentration of specific IgA anti-*Salmonella* and anti-HagB increased to 42.9 and 67.4 SFC/ 10^6 MNC, respectively.

The isolation of viable cells from the lamina propria was more difficult and only two groups yielded viable cells for the ELISPOT assay. High numbers of IgA SFC were detected in lamina propria against both *S. typhimurium* (93.5) and HagB (21.5). Once again, boosting at week 14 resulted in an increase; the specific IgA anti-*Salmonella* level increased to 164.5 SFC/ 10^6 MNC and the specific IgA anti-HagB level increased to 81.5 SFC/ 10^6 MNC.

CHAPTER 4 CONCLUSIONS AND DISCUSSION

Reconstruction and Characterization of the Recombinant *S. typhimurium* Strain χ 4072/pDMD1 Expressing the *hagB* Gene of *P. gingivalis*

The use of a live, attenuated, recombinant *Salmonella typhimurium* for delivery of an expressed, heterologous gene is one approach to oral immunization. *Salmonella*'s specificity for targeting the Peyer's patches of the gut provides the appropriate presentation and stimulation to induce mucosal immunity. At the same time, it is capable of inducing systemic immunity. As reviewed earlier, several attenuated forms of the *S. typhimurium* have been developed and investigated as recombinant vaccine strains. In these studies we used the strain χ 4072 which has an *asd* deletion which can be complemented by the *asd* gene encoded on the plasmid pYA292. This "balanced-lethal" construct, developed by Dr. Roy Curtiss, III (Washington University), provides a convenient mechanism for ensuring plasmid stability without the need for continuous antibiotic selective pressure. The requirement for co-administration of an antibiotic is not desirable in a practical vaccine. Our vaccine strain expresses the *hagB* gene, which encodes a hemagglutinin of *P. gingivalis*, cloned into plasmid pYA292 to yield pDMD1 (53).

Although several recombinant constructs of *S. typhimurium* have been reported, expression of the heterologous gene and its effect (e.g., toxicity) on the host strain can vary with each foreign gene. Consequently, it is necessary to characterize the level of expression and resultant growth rate for each construct. Following the reconstruction of *S. typhimurium* χ 4072/pDMD1, we investigated the level and stability of expression of the HagB hemagglutinin as well as the growth rate and colonization potential of the recombinant construct. Through restriction analysis of plasmid minipreps we confirmed

the stable maintenance of the *hagB* gene insert in the plasmid pYA292. From cell lysates of the recombinant strain χ 4072/pDMD1, we identified strong expression of HagB by both Coomassie Blue staining and Western blotting. While high expression of a cloned gene is generally desired for immunogenicity, over-expression could have a deleterious effect on the carrier strain. The expressed protein itself may be toxic to the bacterial host or may consume important resources required for expression of its own critical genes (182, 211). Expression of late genes, or stress-response genes activated *in vivo*, are particularly vulnerable to suppression resulting from over-expression of a cloned gene. For example, the *spv* genes encoded by *Salmonella typhimurium* on a virulence plasmid increase the growth rate of the bacteria within host cells (81), particularly in macrophages (82), and most-likely are activated *in vivo*. While our attenuated strain does not contain the virulence plasmid, other genes may be turned on by *in vivo* signaling and be susceptible to over-expression of our cloned *hagB* gene. In fact, one study identified as many as 34 from a pool of 57 detectable proteins synthesized by *S. typhimurium* that appeared to be unique to the intracellular environment (21).

LPS silver staining confirmed a smooth LPS, again, which is favorable for vaccine strains (76). Rough strains of *Salmonella*, lacking the repeating oligosaccharide units, have been shown to have highly reduced pathogenicity in mice (179, 180) and conventional piglet studies (179). It is postulated that smooth strains may survive longer in the host due to a reduced ability of immune components to readily attach to their surfaces. Again, even though the parent *S. typhimurium* χ 4072 strain had smooth LPS, the expression of HagB could have affected this status.

The effects of the heterologous expression of HagB on the attenuated *Salmonella* were investigated further. *In vitro* growth rate was determined for the recombinant strain χ 4072/pDMD1. A reproducible rate with a doubling time of ~60 minutes during logarithmic growth was determined from three successive studies. This growth rate was slower compared to the control strain χ 4072/pYA292, which had an average doubling time

of ~40 minutes. A decrease in the growth rate of a recombinant strain is common. A critical factor is whether this decreased growth rate would inhibit normal colonization and immunogenicity *in vivo*, which can only be determined by a preliminary study in an animal model. We previously demonstrated the ability of *S. typhimurium* χ 4072/pDMD1 to induce immunity to HagB (52).

In this study, we have demonstrated that the HagB expression in the recombinant *Salmonella* strain is stable even after passage through the BALB/c mice. Colonies recovered from the Peyer's patches five days following oral immunization with the recombinant *S. typhimurium* χ 4072/pDMD1 were consistently positive (100%) for HagB expression by colony immunoblotting. While developing the colonization studies, we discovered that the recombinant strain recovered from the host tissues was more sensitive to selective medium components than the control strain, χ 4072/pYA292. In fact, the use of MacConkey agar, a standard selective medium used in colonization studies of wild-type strains, was found to be too harsh for strains expressing HagB (Fig. 7). It is possible that expression of HagB may affect the outer membrane, making the strain more susceptible to bile salts and crystal violet, which are used as selective agents in this medium. This phenomenon offers a caution for other studies involving recombinant constructs and determination of colonization *in vivo*.

Oral Immunization Study in BALB/c Mice with Boosting at Week 14

One of the concerns with using live avirulent carriers such as *Salmonella* has been that induction of immunity to the carrier may preclude its subsequent use for other vaccines or for boosting. In one report, it appeared that prior exposure to the *Salmonella* carrier enhanced the secondary response to the foreign antigen (4). In our system, we saw recall responses to HagB in serum in both IgG and IgA antibodies as well as in saliva, vaginal washes and in fecal extracts, despite the concurrent high anti-*Salmonella* responses also

detected. In particular, high anti-*Salmonella* IgA levels in the gut might be expected to interfere with the colonization and persistence of the vaccine strain. However, this apparently did not occur with the dosage and immunization regimen used in this study.

In saliva, the response after boosting appeared to be minimal when the data were expressed as nanograms of specific IgA anti-hagB per microgram of total IgA (data not shown). This was done to compensate for the variable dilution one encounters in mucosal secretions. However, in saliva, we detected an increase in total IgA after boosting which tended to damp down the response when expressed in this manner. When the results were normalized to specific antibody per unit amylase (whose level should not be altered by immunization) increases could be detected after boosting.

A possible explanation for the increase in total IgA might involve the response to the *Salmonella* carrier. The number of anti-*Salmonella* spot-forming cells (SFC) in salivary glands found in the ELISPOT studies (Table 1) appears greater than the number of anti-HagB SFC, at least during the primary response. After boosting, however, anti-HagB SFC predominate.

Analysis of the serum IgG subclass distribution was performed to infer the T-helper subset associated with the systemic response. Examination of the IgG subclass distribution of anti-hagB antibodies revealed a predominance of IgG2a antibodies. In mice, Th1 cells produce IL-2, IFN γ , and TNF- β and have been demonstrated to induce B cells to secrete IgG2a (202). Th2 cells on the other hand, preferentially secrete IL-4, IL-5, IL-6, and IL-10 and are most effective in providing help for B cell responses in the form of IgG1 and IgE (202). Th1 responses have been also associated with cell-mediated immune and inflammatory responses (152, 203).

We found a similar predominance of IgG2a anti-HagB antibodies in serum samples collected from BALB/c mice nasally immunized with repeated doses of *P. gingivalis* W50 strain in an experiment (data not shown) conducted by Dr. Jeannine Brady and Dr. Emil Kazarov (University of Florida, Gainesville, FL). Both immunizations are directed toward

mucosal inductive sites, which may influence the T-helper type response and consequently the subclass distribution. Immunization with HagB by a strictly systemic route (e.g., subcutaneously, in adjuvant) would help answer questions concerning the role of the nature of the protein, the delivery system, or route of immunization on the subclass response.

In addition, the serum specific anti-HagB responses in the nasally immunized mice were extremely low compared to the strong responses detected from the orally immunized mice. One possible explanation is that the native expression of HagB in the W50 strain may, in fact, be considerably lower than the expression in the recombinant *Salmonella* strain. A significant difference in antigen concentration at the effector sites could result in a decreased induction of a response. In addition, the immunization routes (oral versus nasal) offer an obvious possible explanation for the differences in the responses. In our mouse studies, however, the different immunization routes most likely do not explain the difference in the anti-HagB response levels since the total serum anti-*P. gingivalis* responses were very high following intranasal immunizations (data not shown). Furthermore, a recent report investigating the kinetics and distribution of immune responses following nasal versus oral immunizations in humans found both routes of vaccination to effectively induce strong serum responses (184).

Nevertheless, the results of our study agree with those of others (162, 213), in which it was found that an antigen expressed in *S. typhimurium* appears to induce a Th1 type immunoglobulin pattern in the systemic compartment. In contrast, it was shown that expression of *S. mutans* Ag I/II as a fusion protein with cholera toxin B subunit induces a mixed Th1/Th2 type immunoglobulin pattern (88). In our studies, a definitive answer will require examination of antigen-specific T-helper cells in both systemic and mucosal sites.

In addition, the implication of a Th1-type association offers the potential for the induction of a cell mediated immune response. This could be explored through a delayed type hypersensitivity (DTH) assay (e.g., footpad or ear pinna swelling). The role of T cells and cell mediated immunity in periodontal disease is still being explored (130), but

there is the potential for a response directed towards specific *P. gingivalis* antigens to help control infection. For example, there is evidence to suggest that *P. gingivalis* may invade epithelial cells (129, 167). Studies of intraepithelial CD8⁺ $\gamma\delta$ T cells in human gingiva suggest that they may have a protective role by cytotoxicity against infected and stressed epithelial cells (124). In another intracellular infection, cell-mediated cytotoxicity appears to play a role in releasing mycobacteria from infected epithelial cells, thus exposing them to other killing mechanisms (99). At the same time an antibody response directed towards appropriate antigens could help eliminate extracellular organisms without inducing excessive inflammation.

Effect of Boosting at Three Different Time Points with Recombinant *S. typhimurium* strain χ 4072/pDMD1 in BALB/c Mice

In this study we investigated the effect of time of boost on the magnitude of a recall response against a cloned hemagglutinin of *P. gingivalis* expressed by an attenuated recombinant *S. typhimurium* strain χ 4072. In the previous study, we had reported a strong recall response following boosting with the same recombinant construct χ 4072/pDMD1 when administered at 14 weeks after the primary immunization; at a time when the primary response in all parameters measured had begun to subside. We investigated the effect of boosting earlier during a strong ongoing primary response at week 7 (Gp I) or at a later timepoint of week 21 (Gp III) as well as at week 14 (Gp II) for comparison. The results suggest that a boost early on during the primary response results in a recall response but with reduced magnitude compared to the response observed previously (109) and in this study with Gp II. In fact, for serum specific IgG and IgA responses in Gp I, one might argue whether the increases compared to the baseline primary response at week 6 are large enough to warrant titling it a recall response.

The serum IgG response seen in Gp III was comparable to that of Gp II. Serum, salivary, and vaginal IgA responses were lower in magnitude in Gp III. However,

compared to the somewhat lower week 6 response, the relative increase at week 21 suggests a recall response.

There are several possible explanations for the reduced recall responses seen in Gp I following boosting at week 7. One theory is that pre-existing gut IgA to the carrier strain, in this case *S. typhimurium* χ 4072, could inhibit the colonization of the same strain for boosting. More specifically, it is thought that a primary response, whether humoral or cell mediated, could prevent normal colonization of *S. typhimurium* and inhibit or reduce the potential of expression of the cloned gene at inductive sites that normally would result in a recall response. This would correlate with the observations seen in this experiment. In addition, the full effect of boosting was observed in Gp II and Gp III which was carried out at timepoints for which the primary response was shown to have dissipated to minimal levels.

Another possibility might be that a refractory period exists in this animal model during which a subsequent boost has little or no effect. This could be due to lack of development of sufficient numbers of memory cells which would be necessary for an enhanced recall response. In fact, antibody tends to inhibit the response of naive B cells and enhance the response of memory cells (66). However, antibody is still present at week 14 when a recall is seen.

Clearly, further studies are needed to clarify which of these theories best explains the reason for the differences in the time of boosting. In fact, it has been suggested in the literature that pre-existing immunity to the carrier strain prevents subsequent use of the same *Salmonella* carrier strain (2). In contrast, Bao & Clements have reported the beneficial effect of prior immunologic experience when using *Salmonella* strains for oral carriers (4). Although we have also demonstrated the effective use of the same carrier strain for boosting at week 14 (109), further studies into pre-existing immunity with boosting at earlier timepoints would not only help to define the observations in this experiment with Gp I, but would also explain the conflicting reports with the ability to use the same strain in

subsequent immunizations, with respect to time. In the next set of experiments, we sought to test the effect of deliberately induced immunity to the carrier on subsequent immune responses.

Effect of Pre-existing Immunity to the *Salmonella* strain on Subsequent Oral Immunization in BALB/c Mice

The use of recombinant *Salmonella* strains for delivery of heterologous genes has been demonstrated in the literature with favorable potential for oral vaccines against a variety of known pathogens (26, 39, 49, 141, 191, 192, 211). Despite these numerous successful reports, one unknown characteristic remains a critical limiting factor for further development of this vaccine strategy; the effect of pre-existing immunity to the *Salmonella* vector strain. Common knowledge of the attributes of the immune system would predict that prior exposure to this vector strain would elicit a memory recall response to the strain, which could limit both colonization of the recombinant *Salmonella* and presentation of the cloned gene products for induction of an immune response. Some studies have in fact shown data to support this theory of carrier-induced epitopic suppression or impairment of immunogenicity due to cross-reacting intestinal antibodies (2, 65). However, the answer to this critical factor is not that clear. Other studies, in contrast, report that prior immunologic experience to the *Salmonella* vector leads to an enhanced subsequent response to the cloned gene (4, 219).

These conflicting reports, together with our earlier findings (109), that oral immunization could induce a recall response in BALB/c mice following boosting with the same recombinant *Salmonella* strain at week 14, was perplexing. From the previous study, we confirmed a time-dependent effect on the potential for recall with a limited response following an early boosting at week 7, coinciding with a strong ongoing primary response. However, we could not determine if this effect was due to pre-existing immunity. In this study then, we set out to systematically determine whether pre-existing

immunity to the *Salmonella* strain was the limiting effect on an early boosting at week 7 in the mouse model.

Our data do not confirm pre-existing immunity to the carrier strain to be a limiting factor. We found minimal effect on the immune responses to the HagB protein induced either in the group of mice with prior immunologic exposure to *S. typhimurium* χ 4072/pYA292 or in the naive group. In addition, we found no significant decrease in the colonization of the recombinant strain in the group with prior immunologic exposure. In fact, this study together with our previous recall response findings (109) further supports the view that pre-existing immunity does not inhibit subsequent use of the recombinant strain.

Our studies, together with the findings of Bao and Clements (4), and Whittle and Verma (219) suggest the route of administration as the explanation for the reported differences. Whereas the majority of the studies reporting suppression have been with an intravenous, intraperitoneal, or subcutaneous route, success in our studies was consistently associated with an oral route (4, 219). Furthermore it has been argued that an oral route is a more meaningful vaccine route for development in humans. Therefore, these findings support the continuing development of recombinant *Salmonella* strains as a significant oral vaccine strategy for induction of protective immunity to many known pathogens.

Pre-existing immunity to the *Salmonella* carrier strain, from our studies in the BALB/c mouse model, does not appear to impair or suppress the potential for induction of an immune response to a cloned gene product. This knowledge will benefit further development of oral vaccines in endemic areas where people have been previously exposed to *Salmonella*.

Long Term Memory and Potential for Recall Response Following Boosting after One Year

Although mucosal immunity has gained much attention in recent studies, the potential for memory associated with a mucosal response or an oral vaccine has remained

unresolved. Early attempts at defining this aspect of oral immunization found no significant long-term memory with peptides or heat-killed preparations (101) compared to the use of live vaccines or the immunogenic adjuvant, cholera toxin (69, 126, 216). It has been reported that in fact the route of immunization, the number of boosts, as well as the use of adjuvants are some of the variables that can affect the time span of memory responses (128, 199). In addition, most studies have been short-term, limiting the ability to define any long-term memory. Clearly, the potential for memory associated with a mucosal response has yet to be defined. In this set of studies, we set out to investigate a potential long-term recall response, and thus implications of a memory response, following boosting with the recombinant *S. typhimurium* χ 4072/pDMD1 at one year.

From this set of studies, which includes three separate groups with varying times of primary immunization or early boost (Gp III), we consistently detected strong recall responses in both systemic and mucosal parameters. In particular, the IgA responses, both systemically and in mucosal secretions, were elevated to higher maximum levels than previously seen with earlier successful boosting regimens at week 14. The reproducibility of the long-term recall responses measured in these three separate study groups of BALB/c mice provides sound and convincing evidence for long-term mucosal memory. Thus, with these studies we have established the precedence for the induction of long-term memory with an oral recombinant *Salmonella* vaccine.

The impact of aging on immune responsiveness, particularly gastrointestinal mucosal immunity, has been the focus of recent studies (190). Some reports would argue that the mucosal immune defenses are compromised in the elderly (6, 132, 215). Still other studies have demonstrated an eight- fold increase (54) in 20- month old rats and a 50% higher level of serum IgA in older than in younger human subjects (1). Steffen and Ebersole also have reported an increased avidity of IgA antibodies with age in mice specific to formalinized *Mycoplasma pulmonis* (200). While total nonspecific IgA levels may increase or remain unchanged with age, it has been suggested that specific antibody titers

may represent a better and more critical measure of mucosal immune responsiveness (54). Clearly, the specific recall immune response, particularly IgA, in our studies increased in magnitude in aged mice. These results indicate that long term memory is induced by our vaccine as evident by the strong recall response. However, the ability to mount a primary response at this age is an issue which needs to be examined separately.

ELISPOT Analysis of Specific Antibody- Secreting Cells from Spleen, Salivary Glands, and Lamina Propria of BALB/c Mice Following Oral Immunization or Boosting

The immune responses induced following an immunization regimen are routinely assessed by measuring specific antibody concentrations in serum or mucosal secretions. The preceding set of experiments utilized ELISA assays to determine specific anti-HagB or anti-*Salmonella* antibodies. Another approach to assess the immune parameters induced following oral immunization or boosting involves an ELISPOT assay. The ELISPOT assay allows for enumeration of, on a gland or organ level, specific antibody-secreting cells. For this study, we utilized the ELISPOT assay to determine the number of specific anti-HagB and anti-*Salmonella* antibody secreting cells localizing to various systemic and mucosal effector tissues following primary oral immunization or boosting. In parallel with other investigations, we analyzed the “spot -forming cells” (SFCs) from spleen, salivary glands, and lamina propria of the gut. Four experiments involving assessment of SFCs after primary oral immunization with the recombinant *S. typhimurium* χ 4072/pDMD1 were performed. We found a 12-fold higher number of IgA versus IgG anti-*Salmonella* SFCs in the spleen. In contrast, we detected 10-100 fold higher levels of specific IgG anti-*Salmonella* antibodies in circulation in our earlier studies. SFC in the spleen may not accurately reflect serum levels of IgG and IgA since many plasma cells secreting antibody after an immune response are found in the bone marrow and lymph nodes and contribute to the total serum pool of antibody.

SFC responses to HagB did increase after boosting in spleen as opposed to responses to *S. typhimurium*. These differences may reflect the fact that the anti-*S. typhimurium* response is being measured against a mosaic of antigens in the whole cell lysate while the anti-HagB response represents the response to a highly purified, single protein. Nevertheless, in mucosal effector sites where IgA responses predominate, we see increases in IgA SFC against both *S. typhimurium* and HagB.

In summary, avirulent *S. typhimurium* has many advantages for induction of an immune response to specific, cloned virulence factors. These vectors are capable of inducing strong mucosal and serum responses as well as recall responses. Multiple epitopes could be expressed if necessary to induce immunity to multiple serotypes or species of periodontal pathogens. The induction of both systemic and mucosal immunity could be of benefit in combating various stages of periodontal disease.

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BIOGRAPHICAL SKETCH

James John Kohler was born in St. Paul, MN, June 30, 1970. He is the elder of two children raised in the "twin cities" area of Minnesota. He completed an A.A. from Anoka-Ramsey Community College as a Presidential Scholar in 1990. He later completed a B.S. with honors in Microbiology and Cell Science at the University of Florida in 1992. He was accepted as a dental student in the College of Dentistry at the University of Florida and began studies in the fall of 1992. After one semester, he transferred into the graduate program in the Department of Oral Biology at the University of Florida to pursue a career in academics. He began work in Dr. Brown's laboratory in May of 1994 after completing laboratory rotations with Dr. McArthur, Dr. Brown, and Dr. Hillman.

The work done on this project has been presented at several national and international research meetings and competitions including the 1995 and 1996 University of Florida Graduate Student Forums, the 24th American Association for Dental Research, the 8th International Congress of Mucosal Immunology, the 1997 University of Florida Medical Guild competition, and the 74th, 75th and 76th International Association for Dental Research Meetings. One publication from this work has been published in Oral Microbiology and Immunology, volume 13, p 81-88, under the title "Oral immunization with recombinant *Salmonella typhimurium* expressing a cloned *Porphyromonas gingivalis* hemagglutinin: effect of boosting on mucosal, systemic and immunoglobulin G subclass response." Three additional manuscripts from this work are in preparation.

James has received a post-doctoral fellowship appointment on the Cancer Biology Training Grant from the College of Medicine at the University of Florida and will be working in Dr. Maureen Goodenow's laboratory beginning in August of 1998.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



Thomas A. Brown, Chair
Associate Professor of Oral Biology

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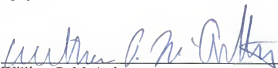
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August 1998



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